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On the Question of Translocation of Heart cAMP-Dependent Protein Kinase

(regulation/cAMP/catalytic subunit of protein kinase/ionic strength effects)

STANLEY L. KEELY, JR., JACKIE D. CORBIN, AND CHARLES R. PARK

Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232

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ABSTRACT **Rat hearts were perfused with epinephrine and/or 1-methyl-3-isobutylxanthine for 2 min. These agents raised the concentration of cAMP and increased the fraction of cAMP-dependent protein kinase (EC 2.7.1.70) in the active form. However, the content of cAMP-dependent protein kinase in the soluble fraction of homogenates of these hearts was reduced and the amount in the particulate fraction was increased. A similar redistribution was obtained by adding cAMP to homogenates of control hearts. The reduction in soluble protein kinase content was due to apparent binding of the free catalytic subunit of the enzyme to particulate material (12,000 × *g* pellet) in media of low ionic strength (<100 mM KCl). The amount bound was, therefore, proportional to the dissociation of the holoenzyme. The binding was not altered by prior boiling or trypsin treatment of the particulate material, but it was prevented or reversed by the addition of 150 mM KCl. The catalytic subunit of the protein kinase from heart also bound to particulate fractions from liver or *Escherichia coli* and to various denatured proteins. These findings suggest that the protein kinase activity of membranes and particulate fractions has frequently been overestimated, since isolation of particulate materials has usually been carried out at low ionic strength. The data also imply that intracellular translocation of the protein kinase catalytic subunit, at least in heart tissue, is of questionable physiological significance.**

There has been much recent interest in the hormonal regulation of adenosine 3′:5′-monophosphate (cAMP)-dependent protein kinases (EC 2.7.1.70) (1–3) and in the protein kinase activity associated with membranes and other particulate fractions (4, 5). We have recently reported the effects of a number of cardioactive agents on cAMP-dependent protein kinase in the perfused rat heart (6). Activation of the heart enzyme takes place according to the equation (7):

$$R_2C_2 \text{ (inactive)} + \text{cAMP} \rightleftarrows R_2\cdot\text{cAMP} + 2\text{C (active)} \quad [1]$$

where R and C represent regulatory and catalytic subunits.

We found that agents or conditions that alter the intracellular cAMP concentration produced corresponding changes in the protein kinase activity ratio, i.e., the ratio of kinase activity in the absence to that in the presence of added cAMP. It was also reported that the total protein kinase activity in the supernatant fraction from hearts was greatly reduced when the activity ratio was high. Other authors have also found a reduction in total kinase activity associated with a rise in the activity ratio and have suggested that translocation of the kinase occurs when cAMP levels are elevated in tissues (8–10). According to our study reported here, total protein kinase activity is reduced because the free catalytic subunit binds to particulate fractions under the usual conditions of study. As a consequence, total protein kinase activity and the activity ratio in particulate fractions have been overestimated in many cases.

Abbreviations: cAMP, adenosine 3′:5′-monophosphate; MeBu^{i}Xan, 1-methyl-3-isobutylxanthine; R and C, regulatory and catalytic subunits of protein kinase.

METHODS AND MATERIALS

Heart Perfusion. Fed male rats weighing 175–225 g were used. The animals were anesthetized with sodium Nembutal intraperitoneally (100 mg/kg). The hearts were quickly excised, immersed in ice-cold saline until beating ceased, attached via the aorta to a perfusion cannula, and perfused with recirculation of the perfusion medium by the method of Morgan *et al.* (11). A filling pressure of 60 mm Hg (8 kPa) was maintained using Krebs-Henseleit bicarbonate buffer at 37° equilibrated with O_2:CO_2 (95:5%) and containing 5 mM glucose and 1 mg/ml of bovine serum albumin. At the end of perfusion, hearts were quickly frozen with Wollenberger clamps which had been cooled in liquid nitrogen, pulverized to a fine powder using a percussion mortar which was also cooled in liquid nitrogen, and stored at −70° until assayed.

Protein Kinase Assay. Approximately 50 mg of powdered heart tissue was suspended at 4° in 0.75 ml of 10 mM potassium phosphate buffer (pH 6.8) containing 10 mM EDTA and 0.5 mM 1-methyl-3-isobutylxanthine (MeBu^{i}Xan) and homogenized with three turns of a loose-fitting Teflon pestle in a glass tube homogenizer. The homogenate was immediately centrifuged at 12,000 × *g* for 20 min at 4°. The protein kinase activity in the supernatant fraction was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP into histone in the presence and absence of added 2 μM cAMP as described earlier (12). The reaction was started by adding 10 μl of the supernatant fraction to 50 μl of a solution containing 17 mM potassium phosphate (pH 6.8), 0.33 mM [γ-^{32}P]ATP (about 33 cpm/pmol), 6 mM magnesium acetate, 0.5 mg of histone, and 2 μM cAMP where indicated. The reaction was terminated by pipetting 50 μl of the reaction mixture onto a 1 × 2 cm filter paper disc which was immediately dropped into cold 10% trichloroacetic acid (10 ml/disc). The filter paper was washed and its radioactivity was measured in 10 ml of toluene–cellusolve scintillation fluid. Protein kinase activity is expressed as units per mg of protein (one unit catalyzes the transfer of 1 pmol of phosphate from ATP to histone in one minute). The protein kinase activity is also expressed as an activity ratio, i.e., the ratio of activity in the absence to that in the presence of added cAMP (2 μM).

Protein. Protein was determined by the method of Lowry *et al.* (13).

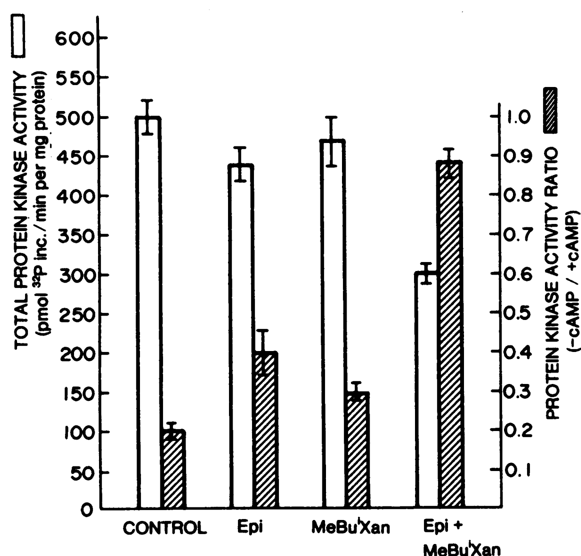

FIG. 1. Effect of epinephrine (Epi) and MeBuⁱXan on the activity and total content of soluble protein kinase. Rat hearts were perfused for 10 min with buffer alone and then for an additional 2 min with buffer containing 0.8 μM epinephrine and/or 10 μM MeBuⁱXan. The hearts were freeze-clamped, powdered, extracted with 10 mM potassium phosphate buffer, and centrifuged at 12,000 $\times$ g for 20 min, and the protein kinase activity was measured in the supernatant fraction as described under *Methods and Materials*. Each bar represents the mean ±SEM of data from three hearts separately. Kinase activity was measured in duplicate.

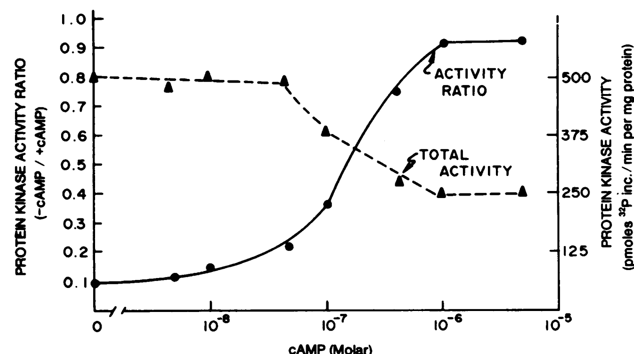

FIG. 2. Effects of cAMP added directly to a crude homogenate. A crude homogenate of a control heart was divided into eight portions and cAMP was added to give the indicated concentration. The portions were then centrifuged at 12,000 $\times$ g for 20 min and the activity ratio and total protein kinase activity were determined in the supernatant fractions.

Materials. Type II-A histone (calf thymus), fraction V bovine serum albumin, horseradish peroxidase, jack bean urease, rabbit muscle aldolase, horse heart cytochrome *c*, and cAMP were obtained from Sigma. [γ^{32}P]ATP was prepared by the method of Glynn and Chappell (14). Epinephrine was obtained as the HCl salt from Parke-Davis Co. MeBuⁱXan was a gift from Searle and Co.

RESULTS

Effects of Epinephrine and MeBuⁱXan on Protein Kinase Activity. When hearts were perfused with either epinephrine or MeBuⁱXan, the protein kinase activity ratio (Fig. 1, hatched bars) increased but the total protein kinase present in the supernatant fraction diminished slightly (open bars). When hearts were perfused with the two agents in combination, the activity ratio rose about maximally to 0.90 but the total activity in the supernatant fraction fell to only 50–60% of that in untreated hearts.

Effects of Exogenous cAMP on the Supernatant Protein Kinase Activity. It was found that the decrease in total protein kinase in the supernatant fraction prepared from hearts with elevated cAMP could also be produced by addition of cAMP directly to the crude homogenate. Fig. 2 demonstrates the effect of adding increasing amounts of cAMP to a homogenate of a control heart. As the activity ratio increased, the total kinase activity in the 12,000 $\times$ g supernatant fraction decreased. When the protein kinase was completely activated (activity ratio >0.90), total activity was reduced by one-half.

Requirement of the Particulate Fraction for the cAMP-Induced Decrease in Supernatant Protein Kinase. When the supernatant fraction of a homogenate was separated from the particulate material prior to addition of cAMP (Fig. 3, bar B), there was little reduction in protein kinase activity compared to the control (bar A). If, however, cAMP was added before the particulate material was removed, there was a 50% reduction in the supernatant kinase activity. This suggested that the free catalytic subunit which would be generated by addition of cAMP (see Eq. 1) was either bound or inactivated by the particulate material.

Binding could be demonstrated, as shown in Fig. 4, when the 12,000 $\times$ g pellet from the heart homogenate exposed to added cAMP was extracted with buffer containing 0.5 M NaCl.* Most of the protein kinase activity lost from the supernatant fraction was recovered (bar B). It can also be seen that salt, added with cAMP before centrifugation, prevented loss of protein kinase activity from the supernatant fraction (bar C). KCl was as effective as NaCl (data not shown).

As is also indicated in Fig. 4, when the 12,000 $\times$ g particulate fractions from control hearts (bar A) were extracted with buffer containing 0.5 M NaCl, only a small, but not necessarily insignificant, amount of protein kinase activity could be extracted. This would suggest that under control conditions little protein kinase is associated with the 12,000 $\times$ g pellet.

The kinase recovered from the 12,000 $\times$ g pellet by extraction with 0.5 M NaCl was the free catalytic subunit of the cAMP-dependent protein kinase as indicated by its elution profile on Sephadex G-100 by the method described earlier (1).

Effect of Ionic Strength on the Binding of the Catalytic Subunit to the Particulate Fraction. To determine the minimum ionic strength needed to prevent the apparent binding of the catalytic subunit to the particulate fraction, various concentrations of KCl, together with cAMP, were added to heart homogenates. As indicated in Fig. 5, concentrations as low as 50 mM promoted recovery of the kinase in the supernatant fraction and loss was completely prevented by 150 mM KCl. The increase in kinase activity above the control level at the latter and at higher salt concentrations suggests that some catalytic

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* Carryover of NaCl into the protein kinase assay slightly inhibits protein kinase activity. However, the inhibition is less than 5% of the total kinase activity measured in the absence of any NaCl.

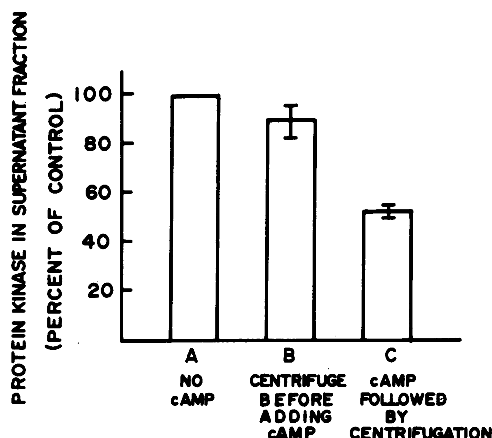

FIG. 3. Effect of the particulate fraction on the protein kinase content of the soluble fraction. Several heart homogenates were each divided into three portions and treated as follows: (A) centrifugation at 12,000 $\times$ g and isolation of supernatant fractions; (B) centrifugation at 12,000 $\times$ g. The supernatant fraction was then isolated and cAMP was added to give a final concentration of 1 μM; (C) cAMP was added to give a concentration of 1 μM. The homogenate was then centrifuged at 12,000 $\times$ g and the supernatant fraction was separated off. Total protein kinase activity was measured in the supernatant fractions. Activity is expressed as the percent of control (portion A), which was assigned a value of 100. Each bar represents the mean $\pm$SEM of three separate experiments.

subunit is bound to the particulate fraction even under control conditions when protein kinase has not been activated by added cAMP.

Binding of the Catalytic Subunit to Other Materials. To determine if the binding of the catalytic subunit of the protein kinase could be prevented by altering the 12,000 $\times$ g particulate material, the pellet formed by centrifugation of a crude heart homogenate was treated by either trypsin or phospholipase, or heated at 100° and then recombined with the supernatant fraction. As seen in Table 1, these treatments had no effect on the binding.

It was also found (Table 1) that the catalytic subunit of the heart protein kinase would bind equally well to the particulate fractions prepared from homogenates of liver or *Escherichia coli*, as indicated by the disappearance of the enzyme from the supernatant fraction after incubation. It can also be seen that the catalytic subunit will bind to some denatured proteins.

DISCUSSION

Our findings suggest that errors in the assay of protein kinase activity in the supernatant and particulate fractions of tissue homogenates may occur because of redistribution of the catalytic subunit in media of low ionic strength. If the ionic strength is below physiological, the catalytic subunit binds to the 12,000 $\times$ g particulate fraction. This binding is proportional to the amount of free C subunit and is inversely related to ionic strength up to 150 mM. Any agent or condition that produces a dissociation of the holoenzyme, RC, would cause an increase in the binding of C to the particulate fraction and thus produce a decrease in the total protein kinase activity in the supernatant fraction and an increase in the particulate fraction. If measurements were made only of the cAMP-independent protein kinase activity, the activating effect of a

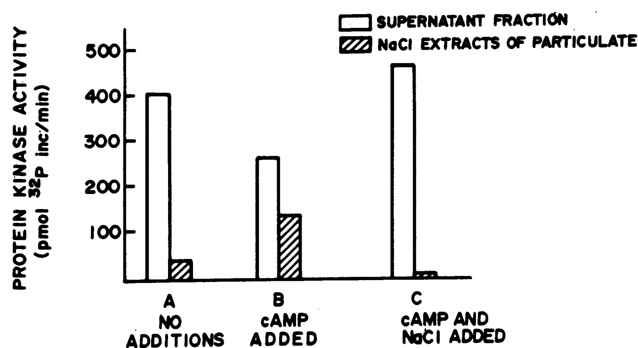

FIG. 4. Prevention by salt of binding of protein kinase to particulate material. A crude heart homogenate was divided into three portions and additions were made as follows: (A) none, (B) cAMP to a concentration of 1 μM, and (C) cAMP as in (B) plus crystalline NaCl to a final concentration of 0.5 M. The samples were centrifuged at 12,000 $\times$ g for 20 min and the total protein kinase activity was determined in the supernatant fractions (open bars). The pellets were resuspended in the original volume of potassium phosphate buffer containing 0.5 M NaCl followed by centrifugation at 12,000 $\times$ g. Protein kinase was determined in the supernate (hatched bars).

particular agent would not be accurately measured, since as much as 40–60% of the free catalytic subunit would be translocated to the particulate fraction. If the protein kinase activity ratio [C/(RC + C)] were measured, the error would be less, since both the numerator and denominator would be affected in the same direction by the decrease in C. The calculated activity ratio, however, would still be somewhat less than the true value, particularly when activity ratios between 0.40 and 0.60 were encountered. In the particulate fraction, translocation would increase total protein kinase artifactually and would give an erroneous impression of a high degree of cAMP independence.

It would seem desirable to employ KCl or NaCl between 100 and 150 mM in homogenization buffers to prevent binding

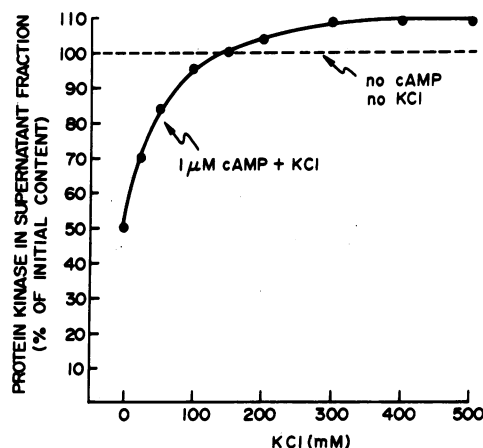

FIG. 5. Effect of ionic strength on retention of protein kinase in the supernatant fraction. A crude heart homogenate was made up to 1 μM cAMP and divided into nine portions. KCl was added to each portion to give the indicated concentrations. The samples were centrifuged at 12,000 $\times$ g for 20 min and the total protein kinase activity in the supernatant fractions was measured. Activity is expressed as the percent of the original activity (before the addition of cAMP) remaining.

TABLE 1. *Binding of heart protein kinase to particulate fractions and denatured proteins*

	Nature and treatment of particulate material added to supernatant fraction	Percent activity remaining in 12,000 × g supernatant fraction
A.	Supernatant fraction alone (no particulate material)	100
B.	+Heart particulate fraction (no treatment)	62 ± 3
C.	+Heart particulate fraction (trypsin-treated)	60 ± 3
D.	+Heart particulate fraction (phospholipase C-treated)	61 ± 3
E.	+Heart particulate fraction (heated)	58 ± 4
F.	+Liver particulate fraction	55 ± 2
G.	+*E. coli* particulate fraction	50
H.	+Denatured bovine serum albumin	76
I.	+Denatured peroxidase	86
J.	+Denatured urease	66
K.	+Denatured aldolase	102
L.	+Denatured cytochrome *c*	87

In B through E, 200 μl of the 12,000 × g supernatant fraction of a crude heart homogenate (containing approximately 200 units of protein kinase activity) were added to 10 mg of the 12,000 × g particulate fraction of a crude heart homogenate which had been treated as follows: (B) no treatment, (C) trypsin (150 μg at 30° for 10 min followed by 300 μg of soybean trypsin inhibitor), (D) phospholipase C (0.5 μU at 30° for 10 min), or (E) heated at 100° for 3 min. In other cases, 200 μl of the same supernatant fraction was added to 10 mg of the 12,000 × g particulate fraction from: (F) rat liver, (G) *E. coli*, or, in (H) through (L), to 10 mg of the indicated protein which had been denatured by heating at 100° for 3 min. The particulate materal was mixed with the supernatant fraction, cAMP was added to give a concentration of 1 μM, and the suspension was centrifuged at 12,000 × g for 20 min. The protein kinase activity was measured in an aliquot of the supernatant fraction. Activity is expressed as the percent remaining from the original supernatant fraction (prior to the addition of any particulate material).

of the catalytic subunit. Higher ionic strength will itself dissociate RC, if the tissue contains the so-called type I cAMP-dependent protein kinase (15).

Jungmann *et al.* (9) reported that elevation of tissue cAMP levels causes the translocation of the ovarian cytoplasmic protein kinase regulatory subunit and catalytic subunit to nuclear acceptor sites. According to Palmer *et al.* (10), stimulation of perfused rat liver by glucagon causes an increase in a nuclear-associated cAMP-independent protein kinase, presumably the catalytic subunit. Korenman *et al.* (8) reported the translocation of uterine muscle protein kinase to the particulate fraction upon treatment of that tissue with isoproterenol and have speculated that the translocation might be responsible for this agent's effect on smooth muscle contraction.

It would be attractive to postulate that translocation of the heart protein kinase is a physiologically significant phenomenon, but the fact that a physiological concentration of salt suppresses the binding *in vitro* suggests that it might be an artifact introduced during homogenization. The fact that heat, trypsin, or phospholipase treatment of heart particulate material does not alter the binding and that the catalytic subunit will bind to particulate fractions from liver and bacteria as well as to various denatured proteins strongly suggests that at least some of the binding is nonspecific. Since the catalytic subunit, unlike the holoenzyme, exhibits a high isoelectric point (14), it might be expected to bind to proteins or other compounds of opposite charge in crude systems. On the other hand, it is important to recognize that the conditions of our study do not duplicate those inside a cell. It is conceivable that certain intracellular conditions could favor binding. One such factor would be the relatively very high concentration of catalytic subunit caused by activation of the holoenzyme within the limited aqueous phase of the cell. The data also do not rule out the possibility that a small percentage of the total catalytic subunit binding, undetectable by the methods used here, could be physiologically meaningful.

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