CHARACTERIZATION OF A CYCLIC AMP-INDEPENDENT PROTEIN KINASE IN THE BOVINE ADRENAL CORTEX

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1. Introduction

The steroidogenic action of adrenocorticotropic hormone (ACTH) on the adrenal cortex is usually considered to be mediated by an increase of intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) and a subsequent activation of cAMP-dependent protein kinase(s) [1–4]. Although the adrenal cortex protein kinase activity has been correlated with the tissue steroidogenic activity [5], several observations have suggested that cAMP and cAMP-dependent protein kinase(s) may not be the sole intracellular pathway involved in the stimulation of corticosteroidogenesis [6–8].

On the other hand, cAMP-independent protein kinases have been described in several mammalian tissues [9,10], although their functions and regulation have remained largely obscure. However, implication of such a protein phosphokinase in liver glycogen metabolism has been recently proposed [11].

This paper reports evidence for the presence in bovine adrenal cortex cytosol of a protein kinase activity which could be characterized as cAMP independent according to the criteria established by Traugh et al. [12]. This type of soluble protein phosphokinase has not previously been described in steroidogenic tissues. The significance of this additional potential pathway in the regulation of adrenocortical functions remains to be established.

Abbreviations: ATP, adenosine triphosphate; TDG buffer, Tris–HCl 10 mM, pH 7.5, containing 0.5 mM dithiothreitol, 5 mM theophylline, 2% glycerol and 0.1 M KCl

2. Materials and methods

Bovine adrenal glands from the local slaughterhouse were processed at 0–4°C within one hour after slaughter of the animals. Adrenal cortex cytosol was prepared as described [13] in ice-cold TDG buffer.

[γ-^{32}P]ATP (2 Ci/mmol) was from Amersham as well as cyclic [8-^{3}H]AMP (36 Ci/mmol) which was checked for purity before use by ion-exchange chromatography on Dowex resin [14]. Unlabeled nucleotides and marker proteins were from Boehringer (catalase mol. wt 240 000; trypsin inhibitor mol. wt 28 000) or Calbiochem (bovine transferrin mol. wt 88 000; egg albumin mol. wt 43 500). [^{14}C]Bovine serum albumin ([^{14}C]BSA) was prepared from BSA (Miles Laboratories) according to Riordan et al. [15].

Protein kinase assay was performed using histone type II A (Sigma) or casein (Merck) under the incubation conditions of Corbin et al. [16] with the trichloracetic precipitation procedure of Sandoval et al. [17].

cAMP-binding activity was measured using nitrocellulose filters (HAWP, 0.45 μm, Millipore) as described by Gilman [18].

Crude preparations of the bovine-muscle protein kinase heat-stable inhibitory protein (HSIP) was obtained by the procedure of Ashby and Walsh [19] up to the trichloracetic acid-precipitation step.

Gel filtration was carried out on Sephadex G-200 superfine (Pharmacia) columns (2.6 × 90 cm) and apparent molecular weights evaluated using the above marker proteins [20].

Sedimentation coefficients were determined [21] by centrifugation in linear sucrose (5–20%) density gradients at 4°C for 21 h at 127 000 × g_{av}.

Protein determinations were performed by the method of Lowry [22] using BSA as the standard.

3. Results and discussion

3.1. *Gel filtration of the crude adrenal cortex cytosol*

Analysis of histone kinase and cAMP-binding activities after filtration of the crude adrenal cortex cytosol through a Sephadex G-200 column yielded the pattern illustrated in fig.1a. The bulk of protein kinase(s) was eluted as a cAMP dependent activity together with most of the cAMP-binding activity. Thus, under these conditions, crude cytosol protein kinase mostly chromatographed as R–C holoenzyme associations [3]. However, the enzymatic activity was clearly heterogeneous and mostly excluded in the filtration process (app. mol. wt > 300 000). This suggested the existence of aggregated forms, as observed in other tissues [23]. In addition, a cAMP-binding moiety was retained on the gel; its app. mol. wt (110 000) was in agreement with reported values for a cAMP-dependent protein kinase receptor subunit [9].

Figure 1b shows the filtration pattern given by the adrenal cortex cytosol previously incubated and chromatographed in the presence of cAMP (2.10^{-6} M). Under these conditions, the major part of the histone kinase activity was retained in the gel (peak II, fig.1b app. mol. wt 55 000), whereas a large cAMP-binding moiety could be isolated (fractions 55–65, fig.1b; app. mol. wt 110 000). In addition, even when larger amounts of cAMP were used, a bulk of protein kinase activity remained mostly excluded from the gel with casein being a better apparent substrate than histone (peak I, fig.1b). No cAMP-binding activity could be detected in these fractions. These gel filtration conditions were used to isolate the two protein kinase moieties which will be further referred to as protein kinase II (PK II) and protein kinase I (PK I), respectively. In addition, the cAMP-binding moiety which was attributed to the R subunit (see below) could be obtained at the same time. The active fractions were

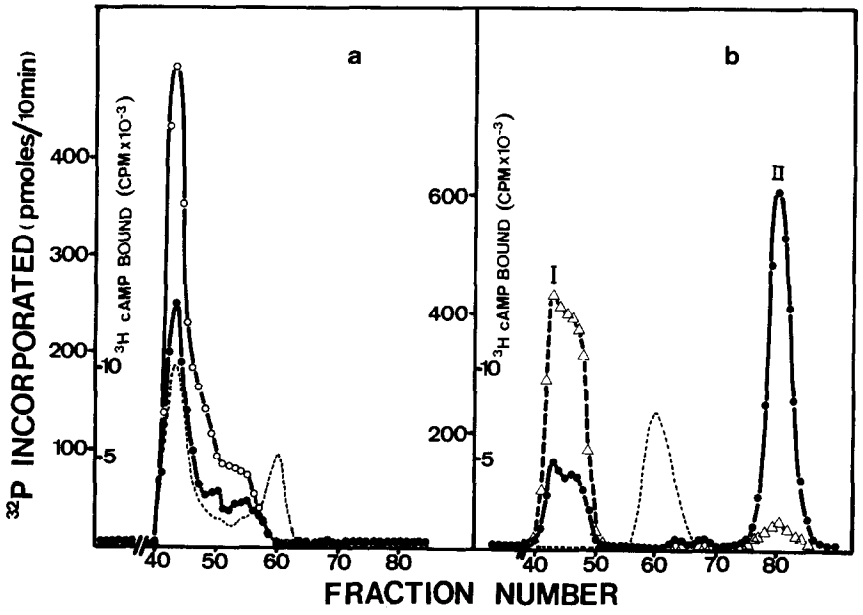

Fig.1. Gel filtration pattern of bovine adrenal-cortex protein kinase activities. Samples (7 ml) of cytosol were applied to a 2.6 × 90 cm Sephadex G-200 superfine column, (a) Crude cytosol applied and eluted in TDG buffer. (b) Cytosol previously incubated with 2.10^{-6} M cAMP and applied to the same column equilibrated and eluted with TDG buffer containing 2.10^{-6} M cAMP. Collected fractions were assayed for histone kinase activity without addition (●——●) and with addition of 1 μM cAMP (○——○), and for casein kinase activity without addition of cAMP (△ - - - △). cyclic [^{3}H]AMP binding activity (- - - - - -) was measured after overnight dialysis against TDG buffer.

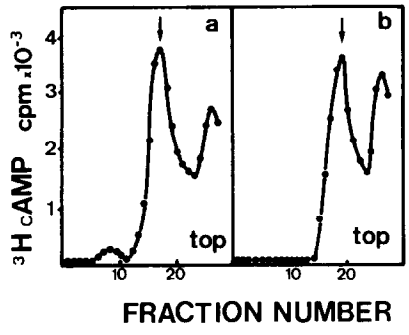

Fig.2. Sucrose density-gradient analysis of adrenal cortex cAMP-binding activity. Pooled fractions (55–65, fig.1a,b) collected after gel filtration were dialysed overnight against TDG buffer, concentrated by ultrafiltration and labeled with cyclic [^{3}H]AMP. Aliquot (200 μl) was layered over a 5–20% linear sucrose gradient in TDG buffer (a) or in TDG buffer containing 0.5 M NaCl (b). Fractions were collected and counted for radioactivity. The arrow indicates the location of [^{14}C]BSA (4.6 S) used as an internal sedimentation marker.

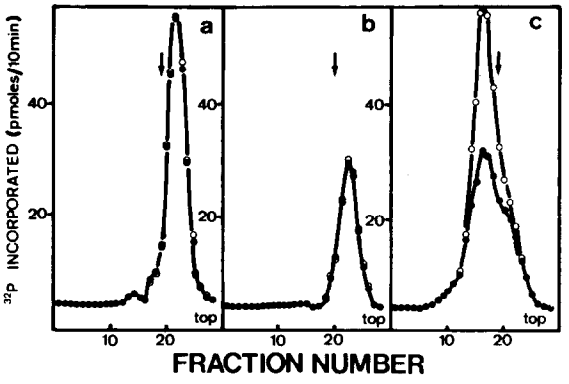

Fig.3. Sucrose density gradient analysis of adrenal cortex protein kinase II. Aliquots (250 μg) of PK II preparation were analysed in 5–20% linear sucrose gradient in TDG buffer (a) and TDG buffer containing 0.5 M NaCl (b). (c) Aliquot of a mixture of PK II preparation (250 μg) incubated with the cAMP-binding protein preparation (160 μg) analysed in a sucrose density gradient in TDG buffer. Fractions were collected and assayed for histone kinase activity in the absence (●——●) or in the presence (○——○) of 1 μM cAMP. The arrow indicates the position of the internal standard [^{14}C]BSA.

filtrated through Sephadex G-25 to eliminate the excess of cAMP and stored at −30°C before use.

3.2. *Sucrose density-gradient centrifugation*

The cAMP-binding moiety isolated after gel filtration was analysed by density-gradient centrifugation after labeling with cyclic [^{3}H]AMP (fig.2). The binding macromolecule exhibited a sedimentation coefficient of 4.6 S (fig.2a). The presence of 0.5 M NaCl did not affect this sedimentation behaviour but suppressed a very minor faster-binding component which may be generated by an aggregation process at low ionic strength (fig.2a). Considering this sedimentation behaviour and the app. mol. wt evaluated by gel filtration (110 000), the cAMP-binding moiety was identified as the receptor subunit of the adrenal cortex cAMP-dependent protein kinase [1]. It may be noticed that a single apparent R moiety could be detected using this methodology, by contrast to the results observed by other workers using ion-exchange separation procedures [24].

3.3. *Protein kinase II identification as C subunit*

Protein kinase II could be identified as the catalytic subunit (C) of the adrenal cortex cAMP-dependent protein kinase with the following observations (fig.3).

(i) Its activity was not affected by the presence of cAMP in the assay and a sedimentation coefficient of about 3.5 S was evaluated with or without salt (fig.3a,b).

(ii) PK II was generated in the cytosol upon addition of cAMP (fig.1b).

(iii) Addition of cAMP receptor protein led to the apparition of a cAMP-dependent activity and a shift of the sedimentation coefficient to higher values of about 5.6 S (fig.3c).

These parameters are in agreement with accepted criteria defining a C subunit [12]. It may be noticed that at low ionic strength, no aggregated form was detected, as observed [1]. On the other hand, our R–C recombination experiments (fig.3c) led to a cAMP-dependent moiety (5.6 S) which may not represent the genuine cytosolic holoenzyme (7 S) [1]. This has already been observed with cAMP-dependent subunits in other tissues [23].

3.4. *Protein kinase I observations*

Protein kinase I as obtained after gel filtration exhibited a sedimentation coefficient of about 7.8 S; addition of cAMP did not affect its activity nor its sedimentation behaviour (fig.4a). Incubation with the

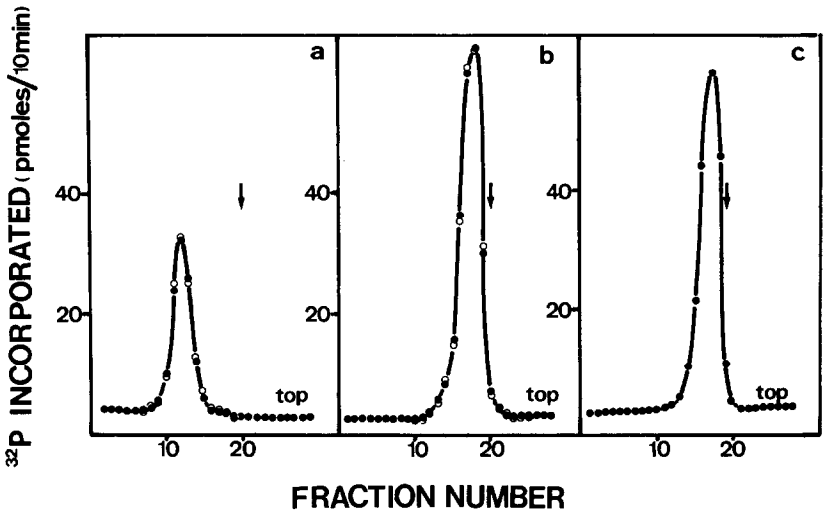

Fig.4. Sucrose density-gradient analysis of the adrenal cortex protein kinase I. Aliquots of PK I preparation (240 μg) was analysed in 5–20% linear sucrose density gradient in TDG buffer (a) and TDG buffer containing 0.5 M NaCl (b) or 0.5 M NaCl + 2 μM cAMP (c). Collected fractions were assayed for histone kinase activity in the absence (•——•) and in the presence (○——○) of 1 μM added cAMP. The arrow shows the location of [^{14}C]BSA.

cAMP-binding subunit (R) had no effect on either parameter and did not induce any cAMP-dependent activity. The cAMP-independent nature of the PK I preparation was further confirmed in separate experiments using various concentrations of cAMP receptor protein preparation (table 1).

By contrast, in the presence of 0.5 M NaCl, the PK I activity was shifted to a slower sedimenting form (5.6 S) and at the same time the specific activity was about three-fold increased (fig.4b). Further addition of cAMP to this salt-activated PK I had no effect either on its catalytic activity or its sedimentation

Table 1
cAMP dependence of PK I and PK II histone kinase activities after incubation with various amounts of cAMP-binding preparation

Protein kinase preparation	cAMP-binding preparation (μg)	Histone kinase activity ^{32}P incorporated (pmol/20 min)	
		– cAMP	+ 1 μM cAMP
PK I 6 μg	0	17.6 ± 0.4	16.1 ± 1.0
	11	16.2 ± 0.7	15.3 ± 0.8
	33	17.9 ± 0.8	20.2 ± 1.5
PK II 6 μg	0	28.9 ± 1.8	27.2 ± 1.1
	11	24.5 ± 1.1	26.2 ± 1.2
	33	16.6 ± 0.7	28.0 ± 1.0

cAMP-binding protein was incubated with either protein kinase I or II preparation for 5 min at 30°C followed by 40 min at 0°C. Histone kinase activities were then assayed after 20 min incubations at 30°C with and without addition of cAMP. Results are mean values from three replicates

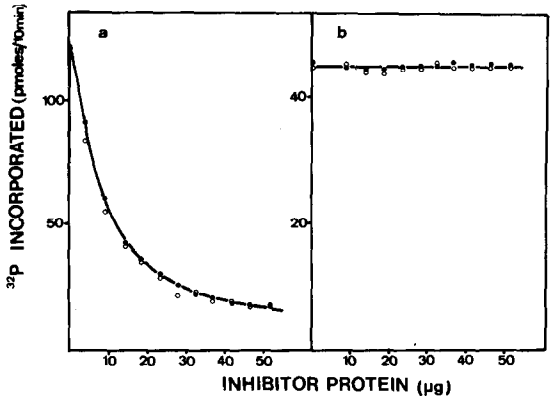

Fig.5. Effect of HSIP on adrenal PK I and PK II activities. Increasing concentrations of beef skeletal muscle HSIP [19] were added to the reaction mixture prior to addition of PK I (a) or PK II (b) preparations. Assays were performed in the absence (●——●) and presence (○——○) of 1 µM cAMP.

behaviour (fig.4c). Independent experiments showed that the enzymatic activity was not affected by various amounts of added cGMP.

3.5. *Effect of HSIP on PK I and PK II*

PK I and PK II histone kinase activities were assayed in the presence of various concentrations of the bovine muscle HSIP preparation (fig.5). PK II activity was inhibited upon addition of HSIP as expected for a cAMP-dependent protein kinase catalytic subunit [12]. By contrast, PK I was not affected by increasing amount of the inhibitor preparation.

4. Conclusion

In addition to the already known cAMP-dependent protein kinase(s) [1] bovine adrenal cortex cytosol was shown to contain a catalytic activity which meets all criteria established for the characterization of a type III, cAMP-independent protein kinase [12]. Casein appeared as a better phosphoryl acceptor than histone for the enzyme. This activity was excluded upon filtration through Sephadex G-200 and therefore exhibited an app. mol. wt > 300 000. It may be suggested that the enzyme is present in the cytosol in association with macromolecular components which may be removed at high ionic strength, resulting in enzyme activation.

Further studies are needed to evaluate the biological significance of this protein phosphokinase activity in the adrenal cortex, particularly with regard to the regulation of steroidogenic functions. In this context it may be of particular interest to consider its possible relationship with the well known cAMP-dependent system and to look for possible specific endogenous substrates.

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