CHARACTERIZATION OF AN ENDOGENOUS SUBSTRATE OF PROTEIN KINASES IN THE BOVINE ADRENAL CORTEX

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1. Introduction

It is currently accepted that the steroidogenic action of ACTH on the adrenal cortex is mediated through an increase of intracellular cAMP and subsequent activation of cAMP-dependent protein kinase(s) [1–4]. Although several observations have suggested that other intracellular processes may be involved [5–7], the adrenal cortex protein kinase(s) activity has been correlated with increased steroidogenesis [8]. In this context, the search for endogenous specific protein kinase(s) substrate(s) may provide a link between protein phosphorylation and key steroidogenic factors. Cholesterol ester hydrolase may represent one of these [9,10]. On the other hand, adrenal microsomal proteins have been suggested as possible target for protein kinase phosphorylation [11,12] whereas in the ovary, a mitochondrial target site involving the cholesterol side-chain cleavage system has been demonstrated [13].

In the course of a study of adrenal cortex protein kinase(s), it was observed that crude preparations of the heat-stable inhibitory protein (HSIP [14]) from this tissue exhibited strikingly different inhibitory properties, as compared to similar preparations from muscle. It was then demonstrated that the crude adrenal HSIP preparations contained an endogenous phosphoryl acceptor as a contaminent. In this paper, we report the characterization of this soluble substrate which can be readily phosphorylated by both adrenal cAMP-dependent and cAMP-independent protein kinases.

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; HSIP, heat-stable inhibitory protein [14]; TDG buffer, Tris–HCl, 10 mM, pH 7.5, containing 2% glycerol, 0.1 M KCl and 0.5 mM dithiothreitol

2. Materials and methods

Bovine adrenal cortex was homogenized in a 10 mM, pH 7.5, Tris–HCl buffer containing 2% glycerol, as previously described [16]. Subcellular fractionation was carried out by sequential centrifugation of the homogenate at 600 × *g* (10 min), 10 000 × *g* (10 min) and 105 000 × *g* (1 h) to yield crude nuclear, mitochondrial and microsomal pellets, respectively, which were washed twice before use. The final supernatant was used as the cytosol.

The substrate activity was found as a contaminent of the HSIP obtained from bovine adrenal cortex following the procedure of Ashby and Walsh [14] up to the trichloroacetic acid (TCA)-precipitation step. After extensive dialysis against TDG buffer, the phosphorylable material was separated from HSIP by gel filtration through a Sephadex G-200 column eluted with TDG buffer.

The catalytic subunit of the adrenal cAMP-dependent protein kinase and a cAMP-independent protein kinase were isolated basically using a gel filtration procedure through Sephadex G-200 in the presence of 2.10^{-6} M cAMP, as described [15]. Protein kinase assays were performed with histone (type II, Sigma) as the substrate, as described by Corbin et al. [17] with the final treatment of Sandoval et al. [18].

Phosphorylation of the endogenous substrate was performed by mixing 50 μl sample with 10 μl [γ-^{32}P]-

ATP solution (8.10^{-5} M, 1000 cpm/pmol in 10 mM, pH 6.8, phosphate buffer containing 9 mM $MgCl_2$) and 20 μl protein kinase preparation.

Linear sucrose (4–20%) density-gradient analyses were run at 127 000 × g_{av} for 16 h at 4°C; [^{14}C]-acetylated bovine serum albumin [19] was added as an internal marker (4.6 S).

3. Results and discussion

3.1. *Evidence for an endogenous phosphorylatable material in the adrenal cortex cytosol*

The crude HSIP preparation from bovine adrenal-cortex homogenate was found

(i) To give a very limited inhibition of the catalytic subunit of the cytosolic protein kinase preparation.

(ii) To stimulate about three-fold the activity of an adrenal cAMP-independent protein kinase activity.

After gel filtration, the HSIP could be clearly separated from a bulk of phosphorylatable material whose presence in the crude HSIP preparation could explain the above observations (fig.1). The phosphorylatable macromolecules were obviously heterogeneous upon gel filtration but exhibited a prominent peak which was isolated and further refered to as the endogenous substrate preparation. Calibration of the gel filtration column (fig.1) allowed the calculation of app. mol. wt 70 000, whereas the HSIP exhibited app. mol. wt 22 000 in agreement with reported values [14].

A possible artifactual nature of the macromolecular substrate components was considered since a rather drastic preparation procedure [14] was followed before the filtration step. However, when crude cytosol was subjected to gel filtration under the same conditions, the eluted fractions corresponding to app. mol. wt 70 000 (substrate retention volume) were found to contain a phosphorylatable material exhibiting similar sedimentation behaviour as that of the endogenous substrate in a sucrose density gradient.

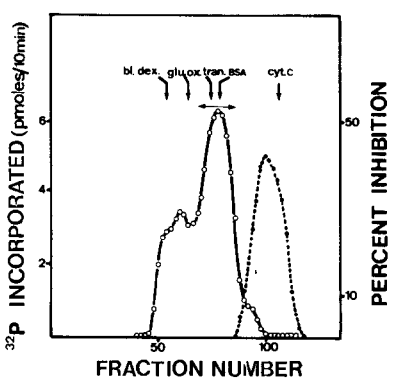

Fig.1. Separation of the adrenal endogenous phosphorylatable substrate from HSIP by gel filtration. The crude HSIP preparation [14] was layered on a Sephadex G-200 column (2.6 × 88 cm), eluted with TDG buffer. Endogenous substrate (o——o) was assayed using an adrenal cAMP-independent protein kinase preparation [15]. HSIP (o– –o) was evaluated by the percent inhibition of histone phosphorylation given by the catalytic subunit of an adrenal cAMP-dependent protein kinase preparation. The arrows indicated the elution volumes of marker proteins: glucose oxidase (glu. ox.); *E. coli* transaminase B (Tran.); bovine serum albumin (BSA); cytochrome *c* (cyt. C); dextran blue (Bl. dex.).

3.2. *Nature of the macromolecular phosphorylatable material*

The endogenous substrate preparation was treated by various proteolytic enzymes, nucleases and a phospholipase A and thereafter analysed by sucrose density-gradient centrifugation. Figure 2 shows that trypsin and pronase totally destroyed the substrate activity whereas DNAase and RNAase did not affect its properties. Phospholipase A partly suppressed the

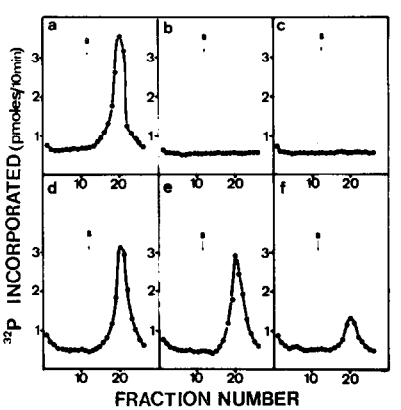

Fig.2. Effect of enzymatic treatments on the endogenous substrate activity. Sucrose density gradient analysis of the endogenous substrate preparation (250 μg protein) before (a) and after treatment (TDG buffer 30 min, 30°C) with 100 μg of (b) trypsin, (c) pronase, (d) DNAase, (e) RNAase, (f) phospholipase A. (B) indicates the location of the internal marker protein ([^{14}C]BSA).

substrate activity, suggesting that a probable protein core may be associated to lipid constituents, although the contamination of the commercial enzymatic preparation by proteases has not been ruled out. A possible lipoprotein nature may be further suggested by the behaviour of the substrate upon density-gradient centrifugation; the macromolecule quickly reached an equilibrium near the top of the 5–20% sucrose gradient while the marker proteins were still sedimenting. As a consequence, the calculation of an accurate sedimentation coefficient was not possible using this method.

3.3. *Subcellular distribution of substrate*

The subcellular distribution of the endogenous substrate was examined after treatment of the individual subcellular fractions following the above preparation procedure. As shown in fig.3, no significant substrate activity could be detected in the particulate fractions, whereas the bulk of the substrate material was found in the soluble cytosolic fraction.

3.4. *Tissue distribution of substrate*

The tissue distribution of the substrate was also considered since its presence in the adrenal cortex and not in other tissues should be a prerequisite for a potential relationship to any specific adrenal cortex function. As shown in fig.4, no substrate activity was detected in bovine liver, muscle and brain. The kidney extract yielded a phosphorylatable material with sedimentation properties quite similar to that of the adrenal substrate, although in lower amount. No substrate activity could be detected in the ovary; however, whole ovaries were treated and it may be worth examining again this point using corpus luteum preparations.

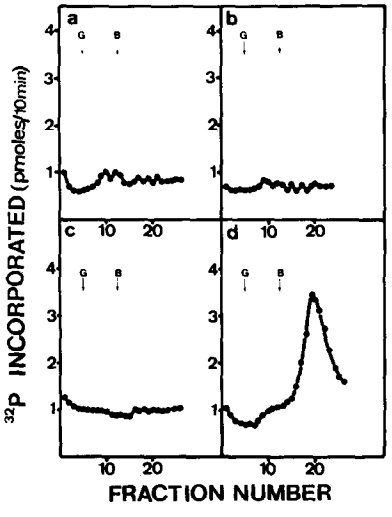

Fig.3. Endogenous substrate activity in bovine adrenal-cortex subcellular fractions. Sucrose density-gradient analysis of the substrate preparations obtained by treatment (see text) of isolated: (a) nuclei, (b) microsomes, (c) mitochondria, (d) cytosol. Glucose oxidase (G) and [^{14}C]BSA (B) were used as marker proteins.

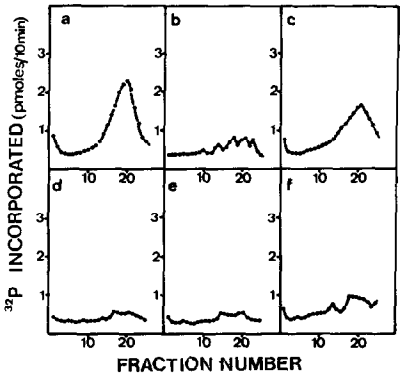

Fig.4. Endogenous substrate activity in various bovine tissues. Sucrose density-gradient analysis of the material obtained following the endogenous substrate preparation procedure (see text) from (a) adrenal cortex, (b) liver, (c) kidney, (d) muscle, (e) brain, (f) ovary.

4. Discussion

A possible biological significance remains to be demonstrated for this endogenous protein kinase substrate. It would be of particular interest to consider its possible implication in adrenal steroidogenic processes and to examine its possible relationship with other heat-stable proteins already described in this tissue, such as a cholesterol binder [20] or a steroidogenic stimulating factor [21], although the latter was also found in adrenal mitochondria.

Acknowledgements

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