CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE DEPENDENT PROTEIN KINASES IN RAT KIDNEY

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1. Introduction

The action of many polypeptide hormones and of catecholamines is at least partly mediated by changes in the level of Ado-3',5'-P in the target cells [1]. The action of parathormone is also mediated by an increase in concentration of Ado-3',5'-P in kidney [2,3]. It has been suggested that the second messenger Ado-3',5'-P acts by activation of specific protein kinases [4] and a widespread occurrence of protein kinase supports this hypothesis [5].

The study reported here shows the existence of at least two Ado-3',5'-P dependent protein kinases in rat kidney.

2. Materials and methods

[γ-^{32}P]ATP (specific activity: 2–10 Ci/mmole) and [^{3}H]Ado-3',5'-P (specific activity: 24.1 Ci/mmole) obtained from NEN-Chemicals. Ado-3',5'-P from Boehringer (Mannheim), histone (calf thymus) from Roth (Karlsruhe), bovine serum albumin from Behring A.G. (Marburg) and DEAE-Sephadex A-25 from Pharmacia (Uppsala, Sweden).

2.1. *Protein kinase assay*

Activity was assayed in an incubation volume of 0.4 ml. The standard assay mixture, unless otherwise indicated, contained 50 mM Tris-HCl, pH 7.4, 8 mM theophyllin, 31 nM Ado-3',5'-P, 2.5 nM $MgCl_2$, 250 μg histone and 175 nM [γ-^{32}P]ATP. The reaction was started by the addition of [γ-^{32}P]ATP. The incubation was carried out for 5 min at 30° in a shaking water bath. The reaction was terminated by the addition of 0.5 ml ice-cold 12% trichloracetic acid and 0.2 ml of 0.5% bovine albumin solution was added immediately.

The technique is then the same as described by De Lange et al. [6]. One unit of kinase activity is defined as the number of pmoles of γ^{32}-P transferred from ATP to the substrate histone in 5 min at 30° per mg protein.

2.2. *Ado-3',5'-P binding activity*

Ado-3',5'-P binding activity was assayed by the method of Wombacher and Körber [7]. The buffer contained 50 mM Tris-HCl, pH 7.4, 8 mM theophyllin, 6 mM 2-mercaptoethanol and 0.25% bovine serum albumin. The unit of Ado-3',5'-P binding activity is defined as the number of pmoles of Ado-3',5'-P bound to 1 mg protein.

2.3. *Protein determination*

The method of Lowry et al. [8] was used to determine protein in suitable diluted aliquots. Electrophoretically pure bovine serum albumin was used as a standard.

2.4. *Procedure*

10 Female wistar rats weighing 150–200 g were fasted overnight, then anesthetized with ether. Their kidneys were quickly removed and chilled in ice-cold buffer medium: 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$.

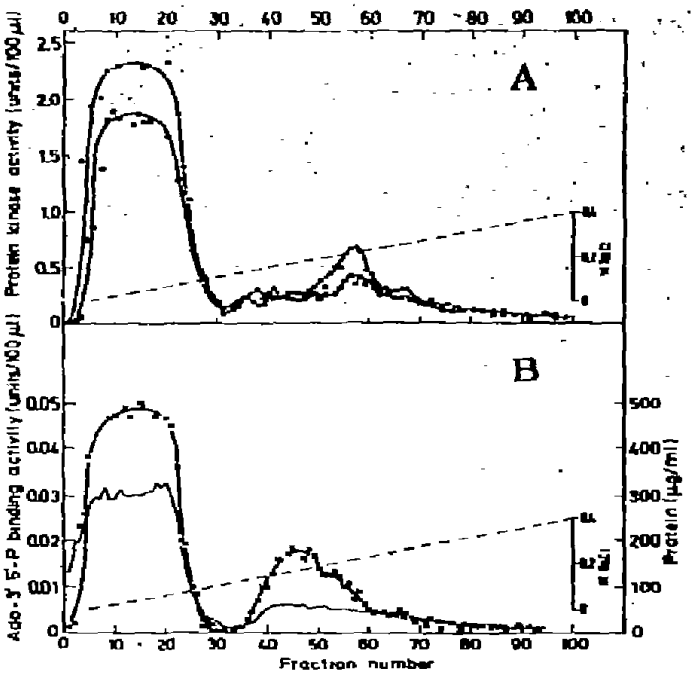

Fig. 1. DEAE Sephadex A-25 column chromatography of protein kinases. A) Protein kinase activity in absence of Ado-3',5'-P (o-o-o). Protein kinase activity in presence of Ado-3',5'-P (•-•-•) (in fractions 1–30: 310 nM Ado-3',5'-P; in fractions 30–100: 31 nM Ado-3',5'-P was used as saturating concentration for activation). B) Ado-3',5'-P binding activity (x-x-x). The column was developed with a linear gradient of sodium chloride (0–0.4 M) in the start-buffer (– – –). Total volume of the gradient was 500 ml, fractions of 5 ml each were collected. Protein concentration (—). Protein kinase activity and Ado-3',5'-P binding activity were determined under conditions described in Materials and methods.

After mincing, the tissue was homogenized in three volumes of ice-cold solution of 0.25 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4 and 8 mM 2-mercaptoethanol with 11 up and down stokes at 800 rpm in a glass homogenizer with teflon pestle (Potter S, Fa. Braun). The homogenate was filtered through two layers of gauze to remove clumps of homogenized tissue, then centrifuged for 10 min at 1475 g_{max} in a refrigerated centrifuge. The pellet was subjected to a subsequent treatment as in Fitzpatrick et al. [9]. This technique yields nuclei, mitochondria and plasma membranes. The supernatant was poured off and centrifuged for 90 min at 145 000 g to produce a microsomal fraction and supernatant.

The microsomal fraction was washed twice with the buffer for homogenization. The microsomal pellet was resuspended by gentle homogenization in a buffer containing 50 mM Tris-HCl, pH 7.4, 6 mM 2-mercaptoethanol, 0.1 mM $MgCl_2$ and 0.5 M NH_4Cl.

The microsomal supernatant was diluted with buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 1 mM EDTA) to a protein conc. of approx. 20 mg/ml, and then applied to a DEAE Sephadex A-25 column [25 cm × 2.6 cm] equilibrated with the same buffer.

3. Results and discussion

The 145 000 g supernatant was resolved on a DEAE Sephadex A-25 column into two peaks of protein kinase activity (fig. 1). As can be seen in fig. 1A and B, the first peak of Ado-3',5'-P binding activity coincides with the first peak of kinase activity, but the second binding peak lies between the two Ado-3',5'-P dependent kinase peaks. Only a shoulder of the second Ado-3',5'-P binding activity peak coincides with the second Ado-3',5'-P dependent kinase peak. The slight kinase activity found between the first and second Ado-3',5'-P dependent kinase peak does not show Ado-3',5'-P dependence.

The above results are similar to those of Gill and Garren [10] found in bovine adrenal glands and those of Tao et al. [11] obtained with rabbit reticulocytes. These authors interpret the results as indicating the presence of dissociable subunits. The enzyme would contain a regulatory (inhibitory) and a catalytic subunit which would dissociate in the presence of Ado-3',5'-P to de-inhibit the activity. This interpretation is also supported by Reimann et al. [12] with rabbit skeletal muscle protein kinase. The evidence presented here indicates the existence of two forms of Ado-3',5'-P dependent protein kinases. They are located almost entirely in the cytosol fraction of kidney.

This supports the unifying hypothesis proposed by Greengard and Kuo [4], that a wide variety of effects, elicited by a second messenger Ado-3',5'-P are mediated through regulation of activity of protein kinases. As at least two Ado-3',5'-P dependent protein kinases can be detected by chromatography on DEAE-Sephadex, the question arises whether isoenzymes initiate the various kidney metabolic functions that are mediated by Ado-3',5'-P. This idea is

Table 1
Ado-3',5'-P binding activity in some kidney fractions (units, as defined in Materials and methods).

Plasma membranes	0.13
Microsomes	0.58
Homogenate, supernatant (30 000 *g*, 1 hr)	1.28
Postmicrosomal, supernatant (145 000 *g*, 1.5 hr)	0.74

discussed for hepatic protein kinases by Chen and Walsh [13] and Kumon et al [14]. The finding of multiple forms of the kidney Ado-3',5'-P dependent protein kinases may lead to a partial explanation of the multiplicity of action of this nucleotide. The increase of renal gluconeogenesis [15] as well as other known effects of parathormone and Ado-3',5'-P suggest that these aspects of kidney metabolism involve the regulation by Ado-3',5'-P dependent protein kinases.

Since histones are very effective substrates of protein kinases from a variety of systems, it had been suggested earlier that a mechanism of control of gene expression might involve Ado-3',5'-P dependent protein kinases. Although it seems that many effects of Ado-3',5'-P are mediated through the phosphorylation of key cellular proteins, the true substrate proteins for Ado-3',5'-P dependent kinases are still

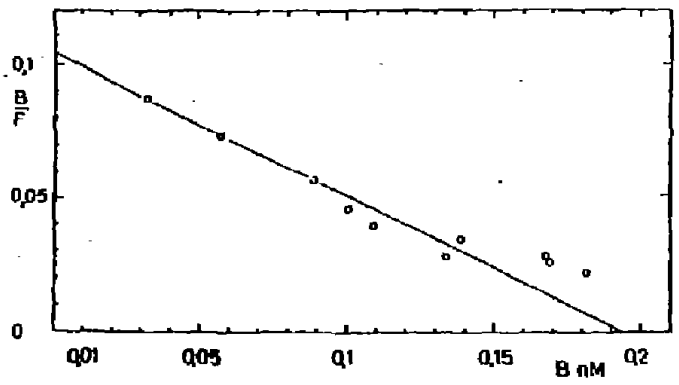

Fig. 2. A Scatchard plot. The amount of nucleotide bound to membranes (B) is plotted as a function of the ratio of the concentration of bound to free nucleotide (B/F). Separation of free Ado-3',5'-P and membrane-bound Ado-3',5'-P and their determination was performed as described earlier [7]. Concentration of binding membrane was 240 μg protein. Affinity constant calculated from slope: $K = 0.53\ nM^{-1}$.

Table 2
Protein kinase activity in some kidney fractions (units, as defined in Materials and methods).

	−Ado-3',5'-P	+Ado-3',5'-P
Plasma membranes	0.92	No difference
Homogenate, supernatant (30 000 *g*, 1 hr)	0.43	No difference
Postmicrosomal, supernatant (145 000 *g*, 1.5 hr)	0.56	0.63
DEAE-Sephadex chromatography		
fraction 8	54.3	65
fraction 56	88.2	143

unknown. Another aspect of Ado-3',5'-P effect is, as can be seen in table 1, the relatively high Ado-3',5'-P binding activity of plasma membrane and microsomes.

A relatively high intrinsic association constant was obtained from a Scatchard-plot (fig. 2).

On the other hand, the plasma membranes possess a high protein kinase activity [table 2], but no stimulation with Ado-3',5'-P is observed, in spite of the relatively high Ado-3',5'-P binding activity.

Another result of potential importance is the increase of protein kinase activity after the separation of microsomes from the homogenate (table 2) and the decrease of Ado-3',5'-P binding activity (table 1). The higher Ado-3',5'-P binding activity of the premicrosomal supernatant homogenate could be related to the high Ado-3',5'-P binding activity of microsomal fraction. Recently, Donovan and Oliver [16] have reported a very high association constant for a protein in ribosomes of rat liver. Therefore the increase of protein kinase activity of the postmicrosomal supernatant could be explained by the higher level of Ado-3',5'-P available to stimulate the protein kinase activity. Finally, Ado-3',5'-P has been implicated in a wide range of actions in the control of kidney function and there are many potential sites for a role for Ado-3',5'-P dependent protein kinases in regulation of kidney function.

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