INTRACELLULAR LOCALIZATION OF ADENYL CYCLASE AND OF BINDING SITES FOR 3′, 5′-ADENOSINE MONOPHOSPHATE IN ADRENAL CORTEX

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1. Introduction

Results concerning the intracellular localization of adenyl cyclase, the enzyme involved in the synthesis of 3′, 5′-AMP, appear to be conflicting. In liver homogenates [1], the plasma membrane fraction displays the highest specific adenyl cyclase activity. In brain, adenyl cyclase is concentrated in the synaptic membranes [2]. On the other hand, there are several reports pointing to the high activity of adenyl cyclase in the mitochondrial fraction of bovine adrenal cortex [3], mouse adrenal tumor [4], rat and dog testis [5,6] and bovine pineal gland [7].

The high adenyl cyclase activity of adrenal cortex mitochondria as found by Hechter et al. [3] is a *priori* compatible with the reported stimulation, by cyclic AMP, of the 11β-hydroxylation in adrenal cortex [8] and with the mitochondrial localization of 11β-hydroxylase [9]. However, since the purity of the mitochondrial preparations obtained by these authors was assessed only by electron microscopy and not on enzymatic criteria, we decided to undertake a more detailed investigation on the subcellular distribution of adenyl cyclase in adrenal cortex. A careful appraisal of the mutual contamination of the different subcellular fractions, based on the activity of specific marker enzymes, led us to conclude, in contrast to the report of Hechter et al. [3], that the adenyl cyclase activity of bovine adrenal cortex homogenates is mainly recovered in the microsomal fraction. The microsomal fraction also displays a much higher number of high affinity binding sites for cyclic AMP than the mitochondrial fraction.

2. Material and methods

The cortex tissue dissected from fresh beef adrenal glands was cut in small pieces and suspend in a cold solution of 0.27 M sucrose, 2 mM tris-HCl pH 7.4; then it was homogenized in a Potter-Elvejhem type glass homogenizer with a teflon pestle at 1,500–2,000 rpm. We used three strokes of a loose fitting pestle and three strokes of a normal clearance pestle. The homogenate was filtered through two and then four layers of cheese cloth to remove connective tissue and large cell debris and centrifuged at 1,000 *g* for 10 min to remove a crude nuclear fraction. The supernatant fluid was centrifuged at 8,000 *g* for 15 min to give a crude mitochondrial fraction. The resulting supernatant was centrifuged at 105,000 *g* for 30 min to give a crude microsomal fraction and a soluble fraction (cytosol). The crude mitochondrial fraction was washed by resuspension in cold isotonic buffered sucrose and sedimented at 9,000 *g* for 15 min. To obtain the purified microsomal fraction, the post-mitochondrial supernatant fluid was first centrifuged at 20,000 *g* for 15 min in order to eliminate contaminating mitochondria or large mitochondrial fragments; then it was sedimented at 105,000 *g* for 30 min.

Cytochrome oxidase was assayed according to Appelmans et al. [10] at pH 7.2 and 25°. Monoamine oxidase was measured with ^{14}C-tryptamine according to the method of Wurtman and Axelrod [11] with a modified extraction procedure [12]. 5′-Nucleotidase was measured according to Emmelot et al. [13] with the following medium: 0.05 M tris-HCl, 5 mM $MgCl_2$, 5 mM 5′-AMP, final pH 7.4, total volume: 1 ml; after 20 min of incubation at 25°, the reaction was stopped by 0.2 ml of 30 percent trichloroacetic acid and the inorganic phosphate released was estimated by the method of Fiske and Subbarow [14]. The inhibition of 5′-nucleotidase by nickel ions was tested according to King [15]. The NADPH cytochrome *c* reductase was assayed according to Sottocasa et al. [16] at 25°. To assay the 21-hydroxylase system, adrenal cortex fractions (0.5–4 mg protein) were incubated for 40 minutes at 37° in the following medium: 100 mM tris-HCl pH 7.4, 2 mM NADP, 20 mM glucose-6-phosphate, 2.5 μl purified glucose-6-phosphate dehydrogenase and 0.4 mM 17α-OH progesterone dissolved in dimethyl formamide. The final volume was 1.1 ml. The steroids were extracted by 5 ml chloroform and 17α,21-dihydroxy 20-keto steroids were estimated by the method of Porter and Silber [17], using 17α,21-dihydroxy pregn-4ene-3-20 dione (11 deoxycortisol) for the standard curve.

The adenyl cyclase activity was assayed by the method of Bär and Hechter [18], slightly modified; the incubation medium contained 20 mM tris-HCl, 6 mM $MgCl_2$, 10 mM caffeine, 10 mM NaF, 2 mM α-^{32}P-ATP (4 μCi/sample), 20 mM potassium phosphoenol-pyruvate, pyruvate kinase 0.05 mg/ml, final pH 8.2, total volume 0.2 ml. The reaction was started by the addition of the enzyme fraction (1–3 mg protein). The incubation period (20 min at 25° was ended by addition of 0.05 ml of 30 percent trichloroacetic acid. An aliquot fraction (at least 0.05 ml) of the trichloroacetic extracts was applied together with carrier cyclic AMP and 4 μmoles of EDTA upon a Whatman no 1 paper which had been impregnated with ammonium sulfate as described by Lane [19] for the separation of nucleosides and by Duée [20] for the separation of adenine nucleotides. The separation of cyclic AMP from AMP, ADP, ATP, pyrophosphate and phosphate was achieved with the solvent described by Krebs and Hems [21] modified as follows: isobutyric acid–1 N ammonia–0.4 M EDTA (400:120:1, by volume) [22]. The cyclic AMP spot was located under UV light, cut out from the paper sheet and counted in a low background gas flow counter (Model RA 15, Intertechnique, Paris).

Binding of cyclic AMP to sucellular particles was measured in the following medium: 0.12 M KCl, 0.02 M tris-HCl, 0.01 M potassium phosphate, 0.01 M caffeine, 0.005–250 μM ^{14}C-cyclic AMP, final pH 7.4; the particle fraction (1–4 mg protein) was added to initiate the reaction. The total volume was 2 ml. The incubation was carried out at 0° for 30 min and stopped by centrifuging at 25,000 *g* for 30 min. The radioactivity in the pellet (after digestion of the pellet by 1ml of formamide (at 180°) and in the supernatant fraction was measured by liquid scintillation counting. The amount of bound cyclic AMP was calculated according to Salomon and Schramm [23] by using a "blank" of binding determined in a parallel assay carried out with a large excess (1 μmole) of unlabeled cylic AMP. Results were plotted according to Scatchard [24]. α-^{32}P-ATP and ^{14}C-cyclic AMP were obtained from the Commissariat a l'Energie Atomique, Saclay. France.

3. Results and Discussion

3.1. *Distribution of adenyl cyclase*

In table 1, the distribution of adenyl cyclase in crude subcellular fractions from beef adrenal cortex is compared to that of typical marker enzymes: cytochrome oxidase and monoamine oxidase for mitochondria, stereoid 21-hydroxylase for microsomes and 5′-nucleotidase for plasma membrane. Glucose-6-phosphatase which is a classical marker enzyme for liver microsomes [25] has unfortunately a too low activity in adrenal cortex [26] and the contribution of non-specific phosphatases to the glucose-6-phosphate hydrolysis [27] cannot be accurately estimated. NADPH cytochrome *c* reductase, which is also a microsome marker in liver [28], is present both in mitochondria and in microsomes of adrenal cortex, as shown later in this paper. Although, the highest specific activity of 5′-nucleotidase is found in the microsomal fraction (containing plasma membrane), this activity must be assigned mainly to a non specific phosphatase, since it is not inhibited by nickel ions [15].

As shown in table 1, all crude particulate fractions from beef adrenal cortex contained a significant adenyl cyclase activity. The microsomal fraction had the highest

Table 1
Distribution of various enzymes among subcellular fractions of beef adrenal cortex.

	Protein		Cyt. *c* oxydase		Tryptamine oxidase		21-hydroxylase		5′nucleotidase		Adenyl cyclase	
	mg	%	Sp. act.	%	Sp. act.	%	Sp. act.	%	Sp. act.	%	Sp. act.	%
Whole homogenate	7050	(100)	730	(100)	0.058	(100)	0.48	(100)	5.9	(100)	16.4	(100)
Nuclear fraction	1680	(24)	430	(14)	0.035	(14)	0.24	(12)	2.2	(9)	11.8	(17)
Mitochondrial fraction	2080	(30)	1300	(53)	0.116	(59)	0.51	(32)	5.0	(25)	23.2	(42)
Microsomal fraction	760	(11)	460	(7)	0.110	(20)	1.57	(36)	12.6	(23)	37.8	(25)
Soluble fraction	1750	(25)	30	(1)	0.006	(3)	0.24	(13)	8.6	(36)	Not detected	
Recovery		(90)		(75)		(96)		(93)		(93)		(84)
Purified microsomes	–		220	–	0.095	–	–	–	13.5	–	53.6	–

Four crude fractions were obtained from a beef adrenal cortex homogenate as described in Methods. The percentage recovery of total activities is given in brackets. Specific activities are expressed as follows: cytochrome oxidase in nmoles cytochrome *c* oxidized/min/mg protein; tryptamine oxidase in nmoles tryptamine oxidized/min/mg protein; 21-hydroxylase in nmoles 17α, 21-dihydroxy, 20-keto steroids (11 deoxycortisol) produced/min/mg protein; 5′-nucleotidase in nmoles Pi released/min/mg protein. adenyl cyclase in pmoles cyclic AMP produced/min/mg protein.

specific activity, but the main portion of the total activity was recovered in the crude mitochondrial fraction. No cyclase activity was detected in the soluble fraction.

The removal of the contaminant mitochondria from the crude microsomal fraction by sedimentation at 20,000 *g* for 15 min, (as assessed by the decreased cytochrome oxidase activity) substantially enhanced the specific activity of the microsomal adenyl cyclase (table 1). Conversely, upon repeated washings of the crude mitochondrial fraction which resulted in a significant purification of the mitochondrial preparation, as evidenced by electron microscope examination (fig. 1) and by the increase of the specific activities of cytochrome oxidase and monoamine oxidase (table 2), the specific activities of 5′-nucleotidase, 21-hydroxylase and adenyl cyclase decreased. The specific activity of adenyl cyclase was nearly ten times lower in purified mitochondria than in purified microsomes, a result which strongly argues against a mitochondrial localization of adenyl cyclase in adrenal cortex.

The unexpected distribution of the NADPH cytochrome *c* reductase in adrenal cortex requires some comments. In fact, the specific activity of NADPH cytochrome *c* reductase in purified mitochondria (51 nmoles cytochrome *c* reduced/min/mg protein) (table 2) is four times higher than that found in microsomes (18 nmoles cytochrome *c* reduced/min/mg protein) (table 2). The NADPH cytochrome *c* reductase activity of adrenal cortex mitochondria is easily accounted for the presence in the inner mitochondrial membrane [29] of the *P*-450 hydroxylating system for which cytochrome *c* is an artifical electron acceptor. It must be incidently pointed out that cytochrome *c* is rapidly reduced upon incubation (in the absence of NADPH) with an adrenal cortex homogenate or with derived subcellular fractions. Since heat treatment (3 min at 100°) does not alter the rate of cytochrome *c* reduction, this reduction is likely to be ascribed to endogenous ascorbic acid, which is present in substantial amounts in adrenal glands.

In view of the above mentioned observations, the steroid 21-hydroxylase system appears to be the best enzymatic marker for adrenal cortex microsomes.

3.2. *Binding of* ^{14}*C-cyclic AMP*

The binding of ^{14}C-cyclic AMP was investigated by the Scatchard method [24] with purified mitochondrial and microsomal fractions. Data in fig. 2 indicate that cyclic AMP preferentially binds to the microsomal

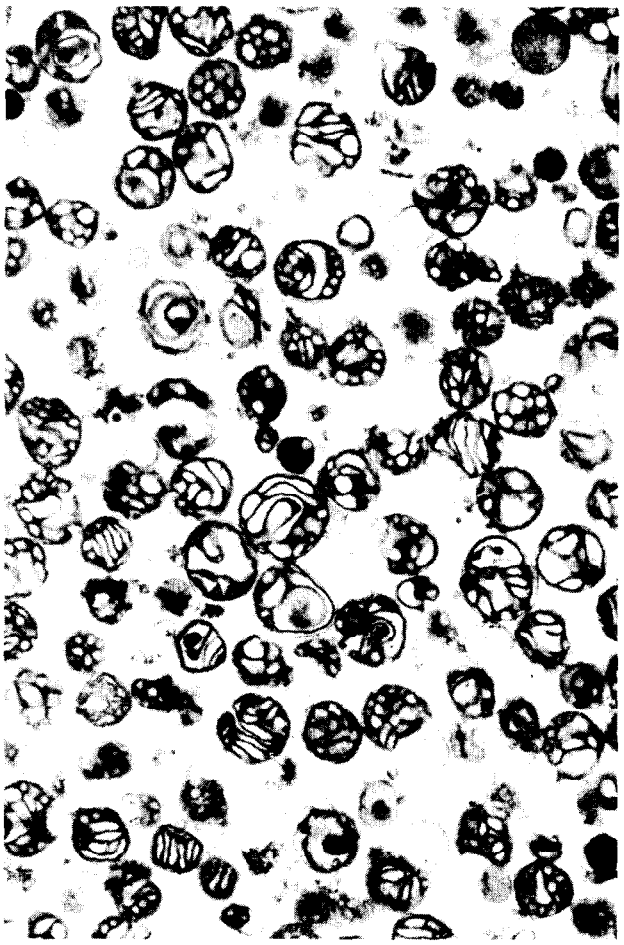

Fig. 1. Thin section of beef adrenal cortex mitochondria, after three washings. Fixation with 1.5 percent glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.2 and then with 2% osmium tetroxide in the same buffer. Embedding in Epon 812. The sections were contrasted with uranyl acetate and lead citrate (X 12,000). The micrograph shows only a slight contamination by extramitochondrial particles. Note that the mitochondria display a condensed conformation.

fraction. In a first approximation two classes of binding sites can be discriminated. The high affinity binding sites (about 1.5 pmoles/mg protein) are characterized by a dissociation constant (K_d) equal to (or lower than) 0.02 μM. The K_d of the low affinity binding sites is higher than 0.2 μM.

To determine the K_d values, we intentionally used a saline medium with a rather high ionic strength, in

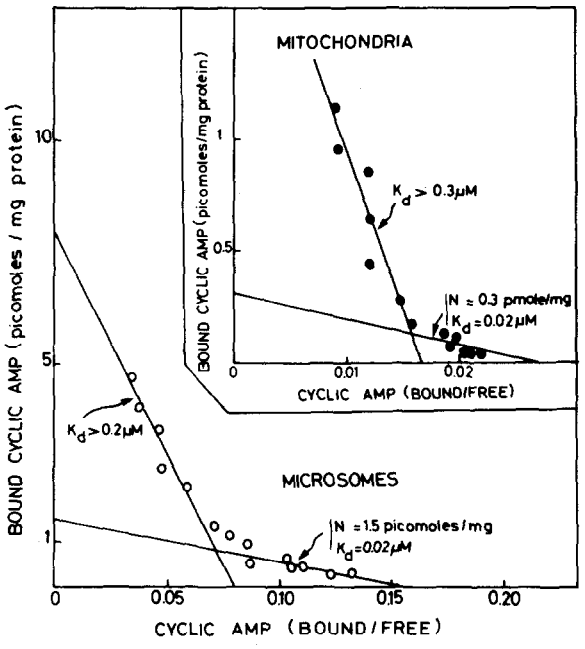

Fig. 2. Scatchard plot of binding of the cyclic AMP to beef adrenal cortex microsomes and mitochondria. Conditions as described in Methods.

order to minimize any ionic effect brought about by different added compounds. The ionic strength effect on the K_d values of the microsomal high affinity bindings sites is shown in fig. 3; a K_d value of 0.8×10^{-8} M can be calculated for an ionic strength extrapolated to zero.

The small number of binding sites for cyclic AMP found in mitochondria (five times less than in microsomes) is mainly accounted for by contaminating microsomes, on the basis of the steroid 21-hydroxylase activity. These results are in agreement with the report by Gill and Garren [30] that cyclic AMP binds specifically to a protein which is predominantly associated with the microsomal fraction of the adrenal cortex cell.

The data reported in this paper can be summarized as follows:

1) The adenyl cyclase activity is present in all crude particulate fractions obtained from homogenates of bovine adrenal cortex.

2) Purification of the crude mitochondrial fraction,

Table 2
Effect of successive washings of crude mitochondrial fraction from beef adrenal cortex on the specific activity of various enzymes.

Mitochondrial fractions	Specific activities					
	Cytochrome oxidase	Tryptamine oxidase	NADPH cyt. *c* reductase	21-Hydroxylase	5'-Nucleotidase	Adenyl cyclase
Crude fraction	1300	0.12	15.2	0.51	5.0	23.2
One washing	2100	0.18	22.8	0.29	4.1	9.2
Two washings	2400	0.20	36.0	0.18	2.9	8.1
Three washings	2800	0.25	51.0	0.07	2.3	6.8

The washing procedure is outlined in methods. Specific enzyme activites were expressed as in table 1; NADPH cyt. *c* reductase was expressed in nmoles oxidized cytochrome *c* reduced/min/mg protein.

as assessed by the increase of the specific activities of cytochrome oxidase and monoamine oxidase, is paralleled by a decrease of the associated adenyl cyclase activity; this point is in contrast to the conclusions of Hechter et al. [3]. On the other hand, the elimination of contaminating submitochondrial particles from the crude microsomal pellet results in a substantial enhancement of the specific activity of adenyl cyclase.

3) The high affinity binding sites for cyclic AMP are essentially located in the purified microsomal fraction.

4) Steroid-21-hydroxylase is more suitable as a marker enzyme of adrenal cortex microsomes than glucose-6-phosphatase and NADPH cytochrome *c* reductase.

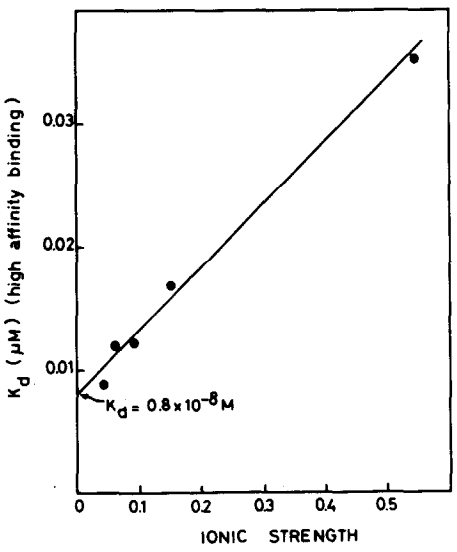

Fig. 3. Effect of the ionic strength on the dissociation constant (K_d) of the high affinity cyclic AMP binding sites. The different ionic strength values plotted in the graph were obtained by modifying the KCl concentration in the basic medium described in Methods.

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