Evidence for two distinct adenylate cyclase catalysts in rat brain

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The Lubrol-soluble adenylate cyclase activity of brain synaptosomal membranes appeared, upon gel filtration or sucrose gradient centrifugation, as two overlapping peaks. Fractions corresponding to the peak of the largest Stokes radius (Biogel pool 1) or highest *s* value (gradient pool 1) contained an adenylate cyclase activity which could be detected whatever the enzyme assay conditions. In contrast, in fractions from the second peak (Biogel pool 2 or gradient pool 2), forskolin was needed to reveal adenylate cyclase activity. The enzyme activity of each Biogel pool was retained by forskolin-agarose and eluted by forskolin with a 34–83% yield. A polypeptide of 155 kDa made up 80% of the forskolin-agarose eluate 1, whereas it was almost absent from eluate 2. Since data from various groups point to the 155 kDa polypeptide as a brain adenylate cyclase catalyst, still another distinct catalyst of lower molecular mass is likely to be present in brain.

(Brain) Adenylate cyclase Enzyme catalyst Forskolin affinity column

1. INTRODUCTION

Eukaryotic adenylate cyclase is a membrane-bound, multi-component enzyme composed of fairly well-known regulatory subunits and of a catalyst [1,2]. Purification of the catalyst did not progress until very recently. Yeast adenylate cyclase has been purified and its gene sequenced [3]; mammalian adenylate cyclase has been partially purified by several groups within the last year [4–8]. One methodology took advantage of the affinity of the brain catalyst for Ca^{2+}-calmodulin and involved the use of Lubrol [4,5], whereas the other relied on its affinity for forskolin and involved the use of Lubro and Tween [6,8]. With the first method, the purified polypeptide was 135–150 kDa, with the second method 116–150 kDa. It is still not known whether the discrepancies in apparent molecular mass are merely due to the use of different detergents, different gel systems and gel calibration in various laboratories, or if they relate to distinct adenylate cyclase catalysts. In this respect, it has long been known that two forms of adenylate cyclase activity exist in the nervous system of higher organisms, one which is stimulated by Ca^{2+}-calmodulin, and the other, which is independent of Ca^{2+}-calmodulin [9,10]. Besides, the existence of mutant files having defects in the calmodulin-dependent adenylate-cyclase activity, but not in the calmodulin-independent activity, favours the view that there is more than one species of adenylate cyclase catalyst [11,12].

Here, we show that gel filtration and sucrose density centrifugation of Lubrol-soluble brain adenylate cyclase allow one to distinguish two forms of enzyme activity which differ by their Stokes radii and *s* values, and by their regulation.

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2. MATERIALS AND METHODS

Materials and methods were as described by D'Alayer et al. [13] and Coussen et al. [4], with minor modifications. Briefly, rat brain synaptosomes were prepared by flotation and lysed once. All preparative buffers lacked metal chelators. Membranes were solubilized with Lubrol (0.9%). The supernatant was layered on a Biogel A5m column (2.8 mg protein/ml) (7 ml) or on sucrose density gradients (0.12 ml), as described [13]. The elution buffer or sucrose gradient solutions were identical, except for sucrose (buffer A: 50 mM triethanolamine, pH 8.0, 0.05% Lubrol PX, 1 mM $MgCl_2$, 2 mM EDTA, 75 mM sucrose, 0.1 mg/l leupeptin and aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol). Gradient and column fractions were assayed for protein, and for adenylate cyclase activity in the presence of 0.1 mM forskolin, 10 mM $MgCl_2$ and 2 mM $MnCl_2$, or in the presence of 0.2 mM EGTA, 0.25 mM $CaCl_2$, 10 mM $MgCl_2$ and 2 mM $MnCl_2$, and 0 or 1 μM calmodulin, as in [4].

On the basis of the calmodulin dependence of the enzyme, the Biogel eluate fractions were pooled: Biogel pool 1 (ml 26 to ml 45), containing most of the calmodulin-sensitive adenylate cyclase activity, and Biogel pool 2 (ml 52 to ml 62), mostly containing calmodulin-insensitive activity. Each pool, brought to 0.4 M NaCl, 1 mM $MgCl_2$, 1 mg/l aprotinin and 1 mg/l leupeptin, was incubated overnight at 4°C with 1.5 ml forskolin-agarose on a roller. The slurry was poured into a column, and the resin washed with 100 ml buffer B (same buffer as above, complemented with 0.5 M NaCl), 100 ml buffer C (buffer B complemented with 2 M NaCl) and 10 ml buffer B. Adenylate cyclase was eluted by incubating the resin at 4°C, 4 times consecutively, 30 min each, with 4 ml buffer B containing 0.1 mM forskolin. Fractions were assayed for adenylate cyclase activity and precipitated with 15% cold trichloroacetic acid. The pellets were washed with cold acetone, dissolved and subjected to PAGE as described [13], except that 7.5–12% acrylamide gels were prepared according to Neville [14]. Gels were stained by the silver method [15] or with Coomassie blue, and scanned. The protein content of the scanned bands was obtained by comparison with bands corresponding to known amounts of molecular mass marker proteins analyzed on the same gel.

Adenylate cyclase assays were performed according to Coussen et al. [4] and proteins were determined according to Schaffner and Weissmann [16]. The forskolin-agarose was prepared according to Pfeuffer et al. [7].

3. RESULTS

3.1. *Evidence for two forms of Lubrol-soluble adenylate cyclase activity*

The distribution of adenylate cyclase activity contained in Lubrol-solubilized brain membranes was studied in Biogel eluates and sucrose gradients. It was found to depend on the enzyme assay conditions.

In the Biogel eluate, a peak of enzyme activity was contained in the void volume and, being of low specific activity, was not studied. In the included volume, basal activity (measured in the presence of either 10 mM $MgCl_2$, or 10 mM $MgCl_2$ and 2 mM $MnCl_2$), and activity supported by 1 μM calmodulin (fig.1A) or 0.1 mM guanylyl imidodiphosphate and 0.2 M ammonium sulfate, or 1 mg/ml asolectin (not shown), were present in fractions corresponding to large Stokes radii (10–12 nm) and appeared as a rather sharp peak. In contrast, forskolin-supported activity covered a broader region of the eluate, with a first peak superimposable on the calmodulin-dependent peak, and a second, overlapping one, of smaller Stokes radii (7.5–8.5 nm) and representing 60–65% of the adenylate cyclase present in the included volume (fig.1A). This distribution was not affected when EDTA-washed membranes [4], rather than control membranes, were used, or if the Lubrol concentration of buffers was lowered to 0.01% (not shown). Fractions were pooled as described (section 2). Upon gel filtration on the same column of Biogel pool 1, adenylate cyclase activity was found only in the fractions of largest Stokes radii (10–12 nm); for Biogel pool 2, the activity was found only in fractions of Stokes radii of 7.5–8.5 nm. Each form of adenylate cyclase activity thus appeared to correspond to stable protein associations.

A somewhat comparable distribution of enzyme activity was found in sucrose gradients. Forskolin-assayed adenylate cyclase activity appeared as a

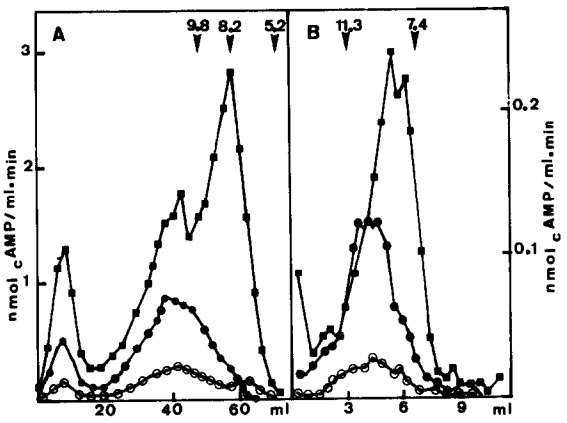

Fig.1. Distribution of adenylate cyclase in the eluate of a Biogel A5m column (A) and in fractions from a sucrose gradient (B). 7 ml Lubrol supernatant (A) and 0.12 ml (B) were analyzed as described in section 2. In A, fractions 1–16 ml correspond to the void volume. Adenylate cyclase activity was measured in the presence of: (■—■) 0.1 mM forskolin, (●—●) 0.1 μM calmodulin and EGTA-Ca^{2+} buffer, as described, (○—○) EGTA-Ca^{2+} buffer only. Arrows indicate: (A) Stokes radius markers (pig thyroglobulin, 9.8 nm; β-galactosidase, 8.2 nm; catalase, 5.2 nm). (B) Sedimentation coefficient markers (catalase, 11.3 S; alcohol dehydrogenase, 7.4 S).

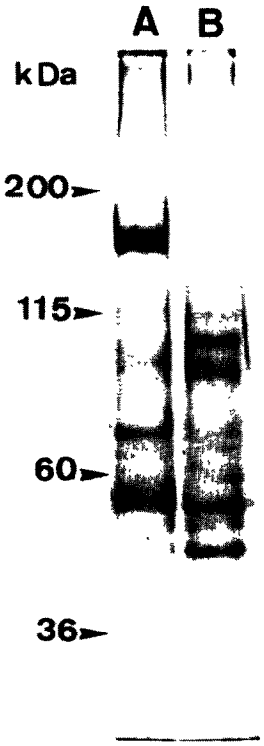

Fig.2. Protein patterns of forskolin-agarose eluates 1 and 2. Aliquots of eluates 1 (A) and 2 (B) containing the same amount of adenylate cyclase activity (2.3 nmol cAMP·min^{-1}) were analysed by PAGE. The gel was silver-stained [15]. Arrows point to molecular mass markers (200 kDa, myosin; 115 kDa, β-galactosidase; 60 kDa, catalase; 36 kDa, glucose-6-phosphate dehydrogenase).

broad peak of sedimentation coefficient 8.8 S, whereas in the absence of forskolin, basal activity and activity supported by 1 μM calmodulin covered a more restricted part of the gradient, in a higher *s* value region (10 S) (fig.1B).

Two forms of adenylate cyclase activity were thus identified by differences in both effector regulation and physico-chemical parameters.

3.2. *Partial purification of adenylate cyclase on a forskolin-agarose column*

Each Biogel pool was incubated with forskolin-agarose, and the adenylate cyclase activity was eluted in the presence of 0.1 mM forskolin, as described. From 34 to 83% of the total activity contained in each Biogel pool applied to the resin was recovered in the corresponding eluate, whatever the Biogel pool (table 1). As analyzed by PAGE, the proteins of eluates 1 and 2 were strikingly different. In eluate 1, one polypeptide of 155 kDa reproducibly constituted 80–100% of the protein content of the fraction, as measured by densitometry of gels stained with Coomassie blue. Low amounts of proteins of 115, 105, 90, 70–74, 52–56 and 44 kDa were also present. In eluate 2, the 155 kDa polypeptide was present in very low amounts or undetectable, and proteins of 115, 105, 90, 52 and 44 kDa were the major components.

Table 1 gives the yield and purification of adenylate cyclase activity achieved, in 4 experiments, for both forms of the enzyme. Since the protein content of the fraction most enriched in adenylate cyclase activity was deduced from densitometry of the gels, the final protein yields and specific activities are only approximate. If the 155 kDa polypeptide present in eluate 1 is indeed the adenylate cyclase catalytic subunit, as proposed [4–6], its specific activity would be 16.8 ± 10 μmol·mg^{-1}·min^{-1}, and its turnover number

Table 1

Purification of adenylate cyclase catalysts

	Protein[a] (mg)	Enzyme total activity[a] (nmol·min^{-1})	Enzyme total recovery (%)	Enzyme specific activity (nmol·mg^{-1}·min^{-1})	Purification (-fold)
Purified brain membranes	39.2 ± 9.1	118.8 ± 39.0	100	3	1
Lubrol-solubilized membranes	35.1 ± 5.3	190.6 ± 32.4	162.5	5.5	1.8
Supernatant	19.6 ± 0.8	158.9 ± 34.5	134	7.6	2.5
Biogel pool 1	2.1 ± 0.2	40.5 ± 5.0	34	18.8	6.2
Eluate 1	0.0016 ± 0.0003	33.6 ± 20.2	28	21000	6930
Biogel pool 2	2.5 ± 0.2	43.7 ± 5.8	37	17.5	5.8
Eluate 2	0.0027 ± 0.0005	15.0 ± 1.4	13	5550	1833

[a] Mean of 4 experiments

Adenylate cyclase activities were measured in the presence of 0.1 mM forskolin

would be about 3000. In eluate 2, the polypeptide responsible for the adenylate cyclase activity was not identified. The specific enzyme activity of this fraction was 5.5 ± 0.9 μmol·mg^{-1}·min^{-1}.

4. DISCUSSION

It has been known for a long time that two forms of adenylate cyclase activity can be distinguished in brain on the basis of their response to Ca^{2+}-calmodulin [9,10]. This study is however the first to show that, upon solubilization, each form of activity is associated with proteins, or protein complexes, of different physico-chemical parameters: *s* values, Stokes radii. These forms did not appear to be interconvertible.

In this study, the calmodulin-sensitive form of activity found in Biogel pool 1 could be activated by guanylyl imidodiphosphate, hence G_s α-subunits were associated with at least some catalytic subunits. The enzyme activity was purified 1000-fold by affinity chromatography on forskolin-agarose, yielding a 155 kDa polypeptide. The final fraction obtained here, and that in a previous study based upon the use of calmodulin affinity chromatography, were comparable in that they both yielded a 135–155 kDa polypeptide as the major component [4]. The specific activity previously attained by us [4] and by Yeager et al. [5] was however much lower than that reported here, suggesting either the copurification of an inhibitor of the enzyme activity, or the partial inactivation of the catalyst. The purification described here represents a considerable improvement with respect to both specific activity and recovery.

The occurrence of another form of enzyme activity, found in a fraction devoid of the 155 kDa polypeptide, suggests that still another type of adenylate cyclase catalyst exists in brain. Could this catalyst derive from the 155 kDa polypeptide by limited proteolysis which would remove the calmodulin-binding and possibly the G_s α-binding domains? Great care was taken to avoid proteolysis all along the purification steps, but this hypothesis cannot be rejected [13]. A further purification of the second form of catalyst is in progress. However, the cloning and sequencing of the cDNA specific for the 155 kDa polypeptide are clearly the next and very important goal to achieve, and should really be crucial in establishing the existence of one or two distinct catalysts.

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