7-*O*-Hemisuccinyl-deacetyl forskolin–Sepharose: a novel affinity support for purification of adenylate cyclase

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1. INTRODUCTION

Hormonally stimulated adenylate cyclase consists of at least 3 individual components: a receptor (R) which recognizes hormones or neurotransmitters; a GTP-binding component (G-protein, abbreviated G, G/F or N); and a catalytic moiety (C) which generates the second messenger cAMP [1–4]. While considerable progress has been achieved in the purification of the G-protein [5–7] and the β-adrenergic receptor [8,9] purification of the catalyst is lagging behind. This may in part be explained by its extreme instability and by the lack of suitable ligands for construction of affinity supports which otherwise proved to be extremely useful for isolation of minute quantities of total cellular protein [9,10]. The most notable success in purification of adenylate cyclase has been achieved with the canine myocardial enzyme by successive hydrophobic and affinity chromatography on ATP–agarose [11]. However, the procedure in [11] failed to resolve C and G since the purified material was still activated by Gpp(NH)p. The first successful separation of the catalyst and the regulatory G-protein has been achieved by affinity chromatography of solubilized pigeon-erythrocyte membrane adenylate cyclase on GTP–Sepharose: While the G-protein was selectively retained by the resin, the catalytic part was exclusively found in the column pass-through [2]. Resolution of G and C by means of gel-filtration in the presence of cholate and high concentrations of ammonium sulfate has been reported independently by two groups [12,13]. Although the two functions were clearly separated by this method, purification of either function was poor.

The hypotensive diterpene forskolin from the roots of *Coleus forskohlii* is a unique and potent stimulator of adenylate cyclase from a variety of sources [14–16]. This plant drug may be specifically directed to the catalytic moiety of the enzyme [16]. This proposal was mainly based on the ability of the diterpene to stimulate adenylate cyclase from the *cyc*⁻ variant of S 49 lymphoma cells [16] known to be functionally deficient in a regulatory G-protein. Furthermore, a water-soluble adenylate cyclase from rat testis, likewise insensitive to guanylnucleotides and fluoride ions, could be stimulated by the diterpene [16]. However, with both the *cyc*⁻ enzyme and the testis enzyme, the extent of stimulation and the apparent affinity for the drug were greatly diminished. On the other hand, low concentrations of forskolin (1 μM) amplified the response of brain adenylate cyclase to several hormones and neurotransmitters. This could be interpreted to mean that the diterpene acts additionally at the receptor or (and) at the G-protein [16].

This study explores the diterpene's potential

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Abbreviations: Gpp(NH)p, guanosine 5′(β-imino)triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl-ether)-*N,N*′-tetraacetic acid

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capability to serve as specific ligand for a purification of the catalytic part of the adenylate cyclase complex by affinity chromatography. Furthermore, a matrix-bound forskolin derivative could be a valuable tool to decide whether the site of action of this compound is exclusively at the catalyst or in addition at another component of the complex. Even the possibility has to be considered that forskolin binds to a yet unidentified component of the adenylate cyclase system.

2. MATERIALS AND METHODS

ATP, Gpp(NH)p, creatine phosphate and creatine kinase were purchased from Boehringer (Mannheim). Succinic anhydride, carbonyldiimidazole, D,L-isoproterenol and Tween 60 were obtained from Serva (Heidelberg). Tween 60 was deionized by passage through a mixed-bed ion exchanger (AG 501 × 8, from Bio Rad, Munich). Lubrol PX was obtained from Sigma (Munich). Sepharose 4B CL and Sephadex G-25 are products of Pharmacia (Uppsala). [α-^{32}P]ATP (10–30 Ci/mmol), c[^{3}H]AMP (15–30 Ci/mmol), NaB^{3}H$_4$ (4.7 Ci/mmol) and [3,5-^{3}H]1-fluoro-2,4-dinitrobenzene (10–30 Ci/mmol) were from the Radiochemical Centre (Amersham). All other chemicals were of the highest purity available. Doubly distilled water was used throughout. Protein was determined as in [17] or, with [^{3}H]fluorodinitrobenzene as in [18].

Soluble adenylate cyclase from cardiac muscle of New Zealand rabbits was prepared as in [19] using 5 mM Lubrol PX in 10 mM MOPS, 1 mM Mg^{2+}/EDTA (pH 7.4) and membrane protein at 10 mg/ml. The Lubrol extract was centrifuged at 200 000 × g for 60 min. A floating turbid layer was discarded and the clear supernatant saved.

Persistently active adenylate cyclase refers to a solubilized preparation derived from myocardial membranes previously incubated with 100 μM D,L-isoproterenol and 100 μM Gpp(NH)p for 10 min at 37°C before solubilisation with Lubrol PX as above. G-protein in the Gpp(NH)p charged form was purified from duck erythrocyte membranes [20] applying a modification of the procedure in [6]. Rat brain adenylate cyclase was solubilized as in [21] with minor modifications.

2.1. *Adenylate cyclase*

Adenylate cyclase assays in the presence of 0.1 mM ATP, unless otherwise stated were performed at 32°C for 10 min as in [2]. cAMP production was linear with time for at least 20 min. Activators at the following final concentrations were added: $MnCl_2$, 2 mM; Gpp(NH)p, 100 μM; NaF, 10 mM; forskolin, 100 μM. Forskolin (or derivatives) were added to the assay mixtures as concentrated ethanolic solutions. Control samples also received ethanol at 0.5% final conc.

Comparable results to these were obtained in at least 3 independent experiments. Standard deviations of triplicate determinations were $<4\%$ of the means.

2.2. *Forskolin–Sepharose*

Forskolin (7β-acetoxy-8, 13-epoxy-1α, 6β, 9α-trihydroxylabd-14-en-11-one, $C_{22}H_{34}O_7$) was converted to deacetylforskolin by alkaline saponification [22]. 7-*O*-hemisuccinyl-7-deacetylforskolin was synthesized by acylation of deacetylforskolin with succinic anhydride in pyridine. Purification and characterisation of the acylation products will be published elsewhere [23]. Aminoethyl–Sepharose 4B CL was prepared by the cyanogen bromide method [24] or by the carbonyldiimidazole method [25]. Aminoethyl–Sepharose was coupled to 7-hemisuccinyldeacetylforskolin via the *N*-hydroxysuccinimide ester according to [26]. To facilitate quantitation of matrix-bound diterpene, a trace amount of NaB^{3}H$_4$-reduced forskolin derivative was added before coupling.

Following hydrolysis with methanolic KOH, 1 ml of packed resin was estimated to contain 0.4–0.8 μmol forskolin derivative. For the sake of simplicity the immobilized diterpene will be designated 'forskolin–Sepharose' in the following.

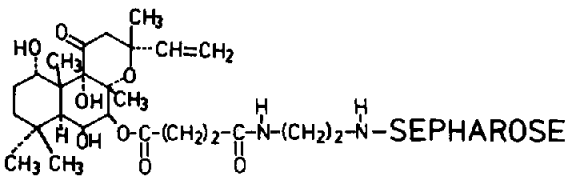

Fig.1. Forskolin–Sepharose 4B.

3. RESULTS AND DISCUSSION

Considering its chemical nature as an oligohydroxy derivative [22] several hemisuccinate derivatives of forskolin have been synthesized. Direct succinylation of forskolin yielded a homogeneous derivative which, however, was barely activating myocardial adenylate cyclase exhibiting a K_{act} at least 2 orders of magnitude higher ($>5 \times 10^{-4}$ M) than the parent compound (3×10^{-6} M). Optimal derivatisation of forskolin resulted from the replacement of the 7-acetyl group by a succinyl group through acylation of deacetylforskolin with succinic anhydride: 7-hemisuccinyl-deacetyl-forskolin exhibited a K_{act} of 9×10^{-6} M with a V_{max} approaching 60% of that of forskolin. This derivative is readily coupled to aminoethyl–Sepharose via its *N*-hydroxysuccinimide-ester.

The experiment in fig.2 reveals that the immobilized forskolin-derivative is indeed able to retain adenylate cyclase activity from solubilized myocardial membranes. The vast majority of total protein applied to the column is found in the pass-through. Moreover, matrix-bound activity can be specifically and rapidly released by free forskolin. While >90% of the activity applied to the column is bound, 35–55% can be recovered by elution with the diterpene following extensive washing with salt and detergent. This one-step procedure yields a 300–500-fold enrichment of forskolin-stimulated adenylate cyclase activity. Although detergent alone is not able to release forskolin–Sepharose-adsorbed adenylate cyclase activity, its presence together with the diterpene in the elution buffer is absolutely required*.

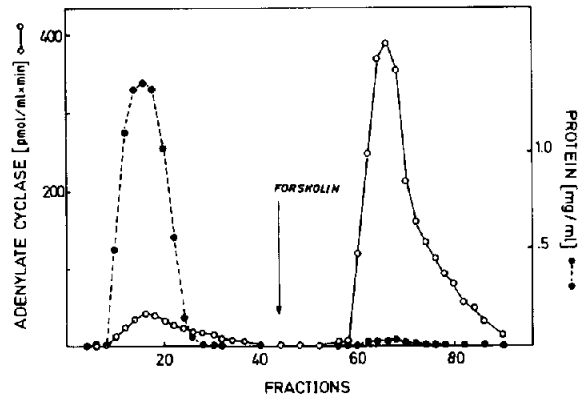

Fig.2. Affinity chromatographic purification of solubilized rabbit myocardial adenylate cyclase on forskolin–Sepharose. Lubrol PX solubilized rabbit myocardial membranes (2 ml, 1.5 mg/ml) were applied to a forskolin–Sepharose column (0.5 × 2 cm) equilibrated with 10 mM MOPS, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM Tween 60 (pH 7.4, buffer A) at 4°C at 1.5 ml/h. The material was washed with another 2 ml buffer A at the same rate. Non-retained protein was washed out with 6 column volumes of buffer A at 4 ml/h. Specifically absorbed material was released from the column by inclusion of 100 μM forskolin into the elution buffer (arrow). Aliquots 20 μl of the fractions (0.2 ml) were assayed for adenylate cyclase activity in the presence of 100 μM forskolin as in section 2.

Stimulation by forskolin of adenylate cyclase from several tissues is readily reversible following washing of treated cells or membranes [15]. We are confirming this with the soluble myocardial enzyme which was depleted from forskolin by gel-filtration on Sephadex G-25. This enabled us to monitor the affinity-purified enzyme for its response to other activators whose actions would have been obscured in the presence of diterpene. The purified enzyme has become refractory to those activators which are known to act through the GTP-binding component (table 1). However, the ratios of Mn^{2+}- or forskolin-stimulated to non-stimulated activities are essentially the same as with the control preparation. The material specifically desorbed from the immobilized diterpene, however, has preserved its ability to interact with the separated G-protein fraction or with a purified G-protein from duck erythrocyte membranes. These data confirm the idea that the action of diterpene is indeed on the catalytic portion of the adenylate cyclase complex.

Forskolin–Sepharose could be successfully applied in recovery of adenylate cyclase from membranes of rat brain cortex [23]. Fig.3 shows different functions of solubilized rat brain adenylate cyclase before and after forskolin–Sepharose chromatography. In accordance with the results obtained with the myocardial enzyme the affinity column efficiently resolves the catalytic part from the proteins required for stimulation by guanyl-

*Desorption of adenylate cyclase activity was carried out in the presence of Tween 60 which is much less inhibitory than Lubrol PX, especially at low protein–detergent ratios

Table 1

Refractoriness of forskolin–Sepharose-purified myocardial adenylate cyclase towards guanyl nucleotides and NaF

Preparation	Adenylate cyclase activity (nmol . mg^{-1} . min^{-1}) in the presence of:					
	None	Mn^{2+}	Gpp(NH)p	NaF	Forskolin	G-protein[a]
Crude, solubilized	0.13	0.53	0.57	0.65	1.56	1.71
Forskolin–Sepharose eluate	59.2	239.1	58.8	53.7	536	497

[a] When reconstitution of activity by the G-protein was measured, 30 ng purified protein (in the Gpp(NH)p-form) from duck erythrocyte membranes were added to the assay mixture

Solubilized myocardial adenylate cyclase (400 μl, 1.45 mg/ml; crude preparation) or 400 μl pooled fractions 60–80 from fig.1 (forskolin–Sepharose eluate) were chromatographed on a Sephadex G-25 fine column (1 × 6.4 cm) equilibrated with 10 mM MOPS, 100 mM NaCl (pH 7.4). For proper control crude preparation was incubated with 100 μM forskolin at 4°C for 60 min before gel-filtration; 10 μl aliquots were assayed for adenylate cyclase activity as in section 2

nucleotides and fluoride. In addition, the brain enzyme has also been desensitized towards Ca^{2+}.

Like the enzyme from other tissues [27], myocardial adenylate cyclase can be persistently activated by pretreatment with GTP-analogues like Gpp(NH)p or GTPγS, especially in the presence of catecholamines. This persistent activation remains after subsequent solubilisation and further fractionation by gel-filtration or sucrose density centrifugation. With the latter technique, using [^{32}P]ADP-ribosylated G-protein [28] and resolved catalytic function, we could show that the persistently activated system represents a G • C complex of the enzyme [3]. It is of interest that this highly active species of adenylate cyclase can still be activated further by forskolin (1.6–2-fold). The concentration of forskolin required for half-maximal stimulation appears to be one magnitude lower for the activated form of adenylate cyclase when compared with the non-activated enzyme (not shown). It was therefore to be expected that the persistently activated form binds also to the immobilized diterpene (table II). If forskolin–Sepharose-purified enzyme is freed from excess forskolin by gel-filtration, two properties distinguish this form of the enzyme from a corresponding preparation but derived from membranes not pre-treated with Gpp(NH)p. It displays high basal activity in the absence of added Gpp(NH)p or activated G-protein, both of which fail to increase the activity further. A plausible explanation for the persistence of the high activity state would be that this form, most likely the G • C complex, became resistant to affinity chromatographic purification on forskolin–Sepharose. Preliminary experiments showed that the affinity-purified enzyme from persistently-activated membranes sediments with a higher $s_{20,w}$-value (8.7) in a sucrose density gradient than the corresponding preparation from native membranes ($s_{20,w} = 7.0$).

Affinity chromatography of preactivated soluble adenylate cyclase is more efficient than with the non-activated enzyme. In the former case, better recovery and higher purification presumably due to improved stability is achieved. A second property of the affinity-purified enzyme from Gpp(NH)p-treated membranes, which differs from the behavior of the crude soluble preparation from which it is derived, is that the purified enzyme has become refractory to further stimulation by forskolin. Tightly-bound diterpene in the enzyme preparation would explain this behavior. In fact, after chromatography on disulfide–Sepharose, stimulation by forskolin (and binding to forskolin–Sepharose) reappears. But final proof requires following the partition of radioactively-

Table 2

Forskolin–Sepharose chromatography of persistently activated adenylate cyclase

Preparation	Adenylate cyclase activity (nmol . mg^{-1} . min^{-1}) in the presence of:		
	None	G-protein[a]	Forskolin
Gpp(NH)p-preactivated	5.1	5.0	8.6
Forskolin–Sepharose eluate	2688	2520	2666

[a] 30 ng purified G-protein (Gpp(NH)p form) from duck erythrocyte in buffer A was added to the assay mixture

Lubrol PX solubilized preparation (1 ml) of persistently (Gpp(NH)p-pretreated) activated myocardial adenylate cyclase was mixed with 0.5 ml packed forskolin–Sepharose (in buffer A, containing 300 mM NaCl) and gently shaken for 120 min at 4°C. The mixture was centrifuged at 1000 × *g* for 2 min and the supernatant removed. After washing the beads with buffer A (3 times, 2 ml each), adenylate cyclase was released by addition of 100 μM forskolin to the slurry and adjusted to the original volume (1.5 ml). After gently agitating for 60 min at 4°C, the slurry was spun down as above and the supernatant removed (forskolin–Sepharose eluate). When assayed in the presence of 100 μM forskolin adenylate cyclase activity of the non-retained material was 15%, while that of the forskolin-released material was 55% of control activity. For control purposes, the starting material was likewise treated with 100 μM forskolin for 60 min at 4°C. Excessive diterpene was removed in each case by gel-filtration of 400 μl aliquots over Sephadex G-25 fine as in table 1. Adenylate cyclase activity was measured in the presence or absence of 100 μM forskolin as in section 2 except that 1 mM [α-^{32}P]ATP served as substrate

labeled forskolin (or a derivative).

This study demonstrates the usefulness of the immobilized adenylate cyclase activator forskolin for selective recovery of the enzyme from solubilized membrane preparations. This novel affinity support is easily synthesized. Moreover, binding and desorption of adenylate cyclase activity are specific resulting in considerable purification in one step. Finally, bound forskolin can be removed from the purified enzyme, thus making possible repeated cycles of chromatography using the same affinity support system. Forskolin–Sepharose can be regenerated by treatment, for instance with urea, with negligible losses of active ligand.

These data indicate that activation of adenylate cyclase by the diterpene results from a direct interaction with the catalyst (or a component closely associated with it). A mechanism requesting the removal of an inhibitory component can be discarded. Further studies must show whether the forskolin binding site is located on the same polypeptide chain which bears the catalytic site. However, the nearly complete lack of a response of adenylate cyclase from duck and pigeon erythrocyte membranes to forskolin activation and the failure of immobilized forskolin to bind to these adenylate cyclases [23], shows that at least in these cases a separate forskolin binding component should be seriously considered.

The forskolin–Sepharose derivative is versatile, since it binds different forms of adenylate cyclase non-activated and persistently; e.g., Gpp(NH)p (or Mg^{2+}/F^{-}) activated enzymes. When non-activated adenylate cyclase is chromatographed, a preparation is obtained which is apparently resolved from the regulatory G-protein (and from calmodulin in the case of the brain enzyme). G-

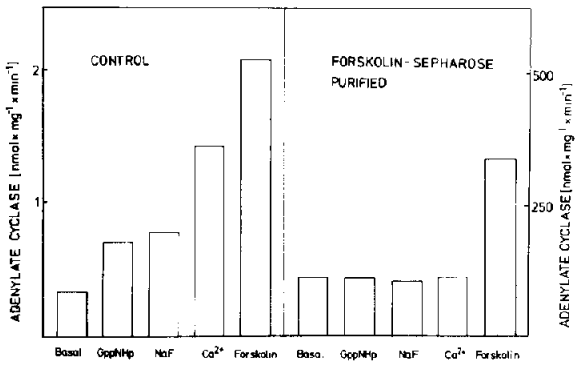

Fig.3. Loss of nucleotide-, fluoride- and Ca^{2+} stimulation of rat brain adenylate cyclase following forskolin–Sepharose chromatography. Solubilized rat brain adenylate cyclase (1 ml, 1.2 mg protein) was mixed with 0.5 ml packed forskolin–Sepharose in buffer A (containing 300 mM NaCl and 0.6 mM EGTA) and gently shaken for 120 min at 4°C. The slurry was then centrifuged at 1000 × *g* for 2 min and the supernatant saved. Following 3-times washing of the resin with 2 ml each of buffer A, containing 0.2 mM EGTA, adenylate cyclase was released with 100 μM forskolin added to the slurry in the original volume (1.5 ml). After gently agitating the mixture for 60 min, the released protein was separated by centrifugation as above (forskolin–Sepharose purified). A control preparation was likewise diluted with the above buffer and incubated in the presence of 100 μM forskolin for 60 min. Forskolin was removed from control and affinity purified fraction by passage over Sephadex G-25 following the protocol in table 1. Adenylate cyclase activity in the presence and absence of the activators was determined as in section 2 with the exception that 2 mM cAMP and 0.2 mM EGTA were present in the assay mixture. When stimulation by Ca^{2+} was assayed, 0.25 mM $CaCl_2$ was added. In a typical experiment, 80% of the adenylate cyclase activity (measured in the presence of 100 μM forskolin) was retained by the affinity column of which ~50% could be recovered by elution with forskolin. Note the different scales in both panels.

protein dependent activities are, however, fully restored on addition of G-protein containing extracts from the same tissue or on addition of a purified regulatory protein from duck erythrocyte membranes.

This novel affinity support for adenylate cyclase should be valuable for both analytic and preparative purposes and should be applicable to large-scale purification of this enzyme.

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