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Affinity labeling of forskolin-binding proteins

Comparison between glucose carrier and adenylate cyclase

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An [^{125}I]iodoazidosalicylic acid derivative of forskolin was synthesized for identification of the diterpene's binding sites on the catalytic subunit of adenylate cyclase and on glucose transport proteins. The affinity label was selectively incorporated into proteins of M_r 40 000–60 000 in membranes from human erythrocytes and from various other tissues. The iodoazidosalicylic acid derivative also specifically labeled the catalytic moiety of adenylate cyclase from rabbit myocardial membranes. However, the structural requirements of the two forskolin-binding sites must be different, since the affinity of the photolabel for the glucose carriers is much higher than that for the cyclase catalyst. Furthermore, the label is readily competed with by D-glucose and cytochalasin B for its binding site on the glucose carrier but not on adenylate cyclase.

Glucose carrier; Adenylate cyclase; Affinity labeling; Forskolin

1. INTRODUCTION

For a certain period of time, the hypotensive plant constituent forskolin was considered as a selective ligand for adenylate cyclases from higher organisms. The original suggestion by Seamon and Daly [1] was that the diterpene should bind to the catalytic portion of that enzyme because a variant cell S49 cyc$^-$, devoid of G_s, was likewise stimulated, although to a lower degree. This was finally confirmed by the isolation to homogeneity of the enzyme from heart and brain [2–4].

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Abbreviations: IASA-forskolin, 3′-iodo-4′-azidosalicylamidoethylamido-7-succinyldeacetylforskolin; SD-forskolin, 7-succinyldeacetylforskolin; G_s, stimulatory guanine nucleotide-binding protein of adenylate cyclase; α_s, α-subunit of G_s; C, catalytic subunit of adenylate cyclase; Gpp(NH)p, guanylyl β,γ-imidotriphosphate; TLC, thin-layer chromatography

However, recent studies have revealed a specific interference of forskolin with other systems, where the participation of cAMP was unlikely, e.g. inhibition of carbachol-stimulated uptake of $^{86}Rb^+$ into PC-12 cells, effect on voltage-dependent K^+ channels in the same cell line and inhibition of the insulin-dependent and -independent glucose transport in various tissues [5–8].

Until now forskolin has only been exploited to tag covalently the glucose carrier of human erythrocytes either by using the diterpene directly as a photoaffinity label (of low efficiency [9]) or recently via its iodoazido derivative [10]. This analog has been shown to bind to the glucose carrier with even higher affinity than forskolin itself. However, no data have been presented thus far on the forskolin-binding site of adenylate cyclase. The present study describes the synthesis of a novel forskolin photoaffinity label, iodoazidosalicylamidoethylamidosuccinyldeacetylforskolin (IASA-forskolin) which was exploited for a comparative study of the forskolin-binding sites of glucose carrier and adenylate cyclase.

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2. MATERIALS AND METHODS

2.1. *Materials*

4-Azidosalicylic acid *N*-hydroxysuccinimide ester and Iodogen were obtained from Pierce; cytochalasin B and phenylmethylsulfonyl fluoride were Serva products. 7-Succinyldeacetylforskolin (SD-forskolin) was prepared according to [2]. $Na^{125}I$ (carrier-free), [α-^{32}P]ATP (600 Ci/mmol) and 1-fluoro-2,4-dinitro[3,5-^{3}H]benzene (10–30 Ci/mmol) were from the Radiochemical Centre, Amersham. All other chemicals were of the highest purity available. Mature rabbit hearts were from Pel freez, Rogers, As. Membranes therefrom were prepared and the complex between catalytic subunit and α_s-protein ($C\alpha_s$) of adenylate cyclase was purified according to Pfeuffer et al. [2], with the exception that activation was by AlF_4^- instead of Gpp(NH)p. Briefly, membranes (20 mg/ml) were incubated with 10 mM NaF, 10 μM $AlCl_3$, 6 mM $MgCl_2$ for 1 h at 0°C before solubilisation with Lubrol PX as described. Human erythrocyte membranes were prepared as in [11] and human platelet membranes according to [12].

2.1.1. Photolysis and SDS-polyacrylamide gel electrophoresis

Samples incubated with [^{125}I]IASA-forskolin were irradiated with a UV lamp (Hanau, type 406 AC) of wavelength 254 nm at a distance of 5 cm for 8 min at 4°C. Irradiated samples were dissociated in the presence of 1% SDS and 1% β-mercaptoethanol for 2 h at 30°C and run on 4–15% gradient polyacrylamide-SDS gels according to Laemmli [13]. Dried gels were autoradiographed using Kodak XAR 5 films and Siemens Titan 2 HS intensifying screens. Molecular mass standards were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa). Protein was estimated according to Lowry et al. [14] or as described by Schultz et al. [15].

2.1.2. Buffers

Buffer A comprised 10 mM *N*-morpholinopropanesulfonate (pH 7.4), 0.2 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 150 mM NaCl and 1 mM Tween 60; buffer B was composed of 5 mM phosphate (pH 8.0).

2.2. *Synthesis of IASA-forskolin*

2.2.1. SD-forskolin *N*-hydroxysuccinimide ester (1)

This was prepared according to our previous procedure [2] by reacting 50 μmol (23 mg) SD-forskolin with 8.6 mg (75 μmol) *N*-hydroxysuccinimide and 15.5 mg dicyclohexylcarbodiimide (75 μmol) in 1.4 ml anhydrous acetonitrile for 2 h at 22°C. Precipitated dicyclohexylurea was removed by centrifugation and the supernate containing **1** was immediately reacted with **2** or stored at 22°C under exclusion of moisture.

2.2.2. *N*-Ethylamino(4-azidosalicylamide) (2)

20 mg 4-azidosalicylic acid *N*-hydroxysuccinimide ester (75 μmol) were dissolved in 200 μl anhydrous dimethylformamide and 15 μl ethylenediamine (225 μmol) added. The mixture was left for 2 h at 22°C and the precipitate formed was removed by centrifugation and washed with 100 μl anhydrous dimethyl formamide. The residue was taken up in a minimum amount of methanol (containing 1% of acetic acid) and chromatographed on a 3 ml silica gel (Kieselgel 60, Merck) column using methanol as eluant. The major UV-absorbing ninhydrin-positive product, **2** (TLC on silica sheets using methanol/2% acetic acid as solvent) was collected, the solvent removed by evaporation and the residue taken up in 200 μl absolute dimethyl formamide.

2.2.3. Azidosalicylamidoethylamido-SD-forskolin (3)

SD-forskolin *N*-hydroxysuccinimide ester, **1** (7.6 mg, 15 μmol), in 540 μl acetonitrile and *N*-ethylamino(4-azidosalicylamide), **2** (7.5 mg, 41 μmol), in 150 μl dimethyl formamide were mixed and left for 24 h at 22°C. Following removal of solvents in vacuo, the residue was chromatographed on 3 ml silicia gel using chloroform/methanol (19:1) as solvent. The photoreactive forskolin derivative was monitored by TLC (chloroform/methanol, 9:1) and the fractions evaporated to dryness. The yield of **3** was 5 mg.

2.2.4. 4-Iodoazidosalicylamidoethylamido-SD-forskolin (4)

5 μl of a 0.5 mM solution of **3** in dimethyl formamide, 50 μl of a 0.1 mM solution of NaI in water, 10 μl carrier-free $Na^{125}I$ (1 mCi in 0.1 M NaOH) and 50 μl of 100 mM phosphate buffer (pH 7.4) were mixed and placed into a glass tube (1 × 7 cm) previously coated with 30 μg Iodogen in 100 μl $CHCl_3$. The reaction mixture was left for 20 min at 22°C and then transferred to a glass tube (0.6 × 4 cm) containing 50 μl of a solution of sodium bisulfide (20 mg/ml). After 2 min at 22°C the solution was extracted twice with 150 μl each of water-saturated ethyl acetate. The combined extracts were dried with anhydrous Na_2SO_4 and then evaporated by a stream of N_2 using a charcoal trap. The residue was taken up in dimethyl formamide to give a 0.5 × 10^{-4} M solution of **4** and kept at 22°C. All manipulations concerning the azidosalicylic derivative were performed under protection from light.

3. RESULTS AND DISCUSSION

Fig.1 depicts the route for the synthesis of IASA-forskolin. It was synthesized from the commercially available starting materials, SD-forskolin and 4-azidosalicylic acid *N*-hydroxysuccinimide ester. The latter was converted to the ethylenediamido derivative which was then coupled to the activated SD-forskolin. The resulting forskolin derivative could be easily iodinated with ^{127}I or ^{125}I in reasonable yields.

Fig.2 shows the results from an affinity labeling experiment with [^{125}I]IASA-forskolin and several membrane preparations analysed by SDS gel electrophoresis. In each case the label identified a specific glucose-binding species, since the radioactivity was drastically displaced by D-glucose (but not L-glucose). Unlabeled forskolin (100-fold excess) was likewise able to compete with the label (not shown). While human erythrocyte membranes displayed the characteristic broad band ranging from 65 to 40 kDa also observed with other labels (e.g. [^{3}H]cytochalasin B [9]), the respective pro-

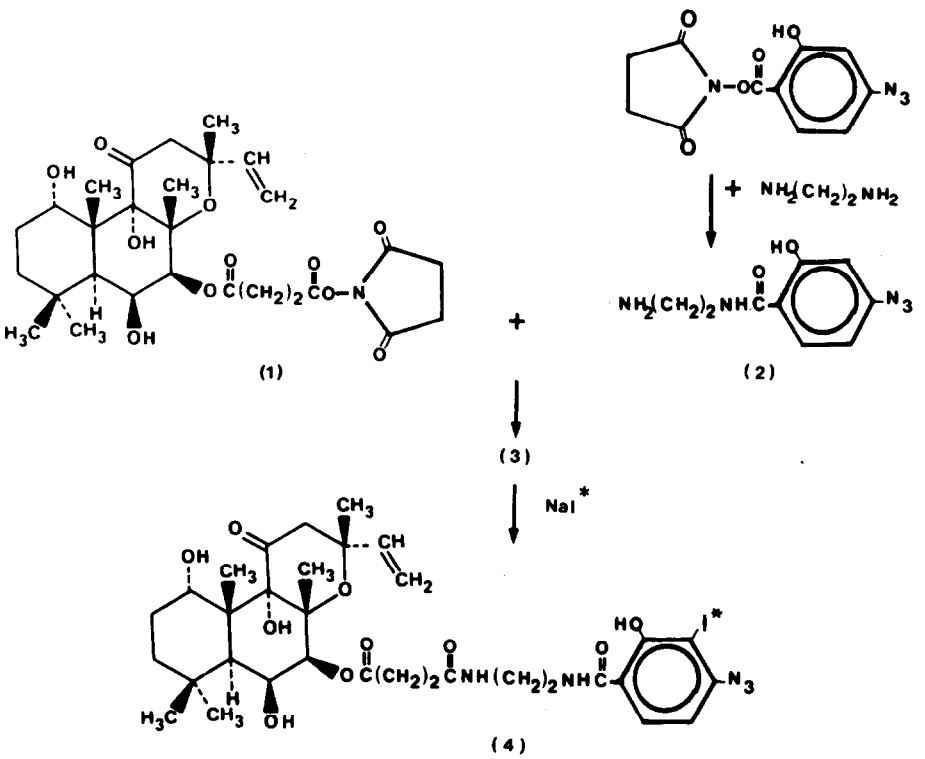

Fig.1. Synthesis of [^{125}I]IASA-forskolin.

teins in rabbit myocardial and human thrombocyte membranes appeared as more discrete bands on SDS gels. When compared to other membranes, including turkey erythrocyte membranes, human red blood cell membranes contain an exceptionally large amount of glucose carrier protein.

Subsequently, we examined [^{125}I]IASA-forskolin with respect to its capacity to serve as an affinity label for adenylate cyclase. Fig.3 shows the dose-response relationship for [^{127}I]IASA-forskolin in comparison with forskolin and the precursor SD-forskolin on activation of AlF_4^--stimulated adenylate cyclase from rabbit myocardial membranes. The activated form was chosen for stimulation and labeling of that enzyme, since there is ample evidence to show that it binds to the diterpene with much higher affinity than the basal (unstimulated) form [16–18]. For example, affinity chromatography on immobilized forskolin is more efficient with the activated form of the enzyme [16]. The latter is considered to represent the complex between the catalyst and the G_s-protein or its α-subunit [2]. Unlike the purified $C\alpha_s$-complex which does bind to forskolin and derivatives, but is not further stimulated, the crude species is still susceptible to activation by forskolin [2,19]. Fig.3 indicates that the affinity label is somewhat less efficient in stimulation of AlF_4^--activated particulate cyclase than the parent compound forskolin but exhibits a similar EC_{50} (0.7 vs 0.3 μM). In contrast, SD-forskolin is much less efficient.

While forskolin seems to bind to glucose carriers and adenylate cyclase with similar affinities, there is a significant difference with respect to the iodoazido derivative (IASA-forskolin). The latter binds to the glucose carrier with even greater affinity (by more than one order of magnitude) than the parent compound. The superior affinity of IASA-forskolin for the carrier allowed for conditions suitable for the suppression of nonspecific binding, e.g. removal of excess label before photolysis by extensive wash steps.

Due to the much lower abundance of adenylate cyclase and as a result of its lower affinity for forskolin and IASA-forskolin, no specific labeling

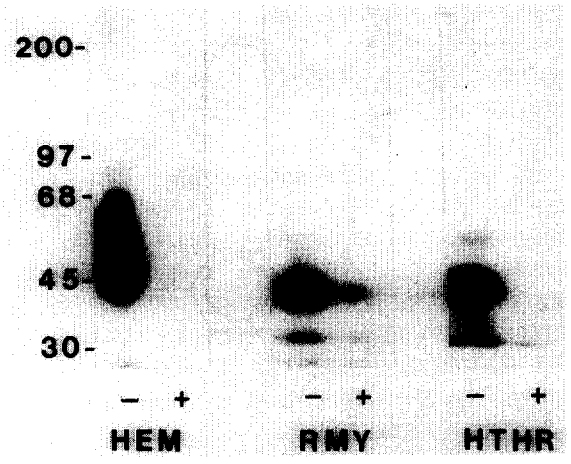

Fig.2. Photoaffinity labeling with [^{125}I]IASA-forskolin of membranes from various tissues. Membranes from human erythrocytes (20 μg), human platelets (165 μg) and rabbit myocardium (205 μg) in buffer A were incubated in the presence (+) and absence (–) of 500 mM D-glucose for 25 min at 4°C. Thereafter, 5 nM [^{125}I]IASA-forskolin (200 Ci/mmol) was added and the mixture incubated further for 25 min at 4°C in the dark. Membranes were then washed three times (2 ml each) with buffer A by centrifugation (50000 × g at 4°C for 5 min). The pellets were dispersed in 150 μl buffer A, irradiated and analysed by SDS gel electrophoresis as described in section 2. Molecular masses (in kDa) are indicated on the left for the standard proteins.

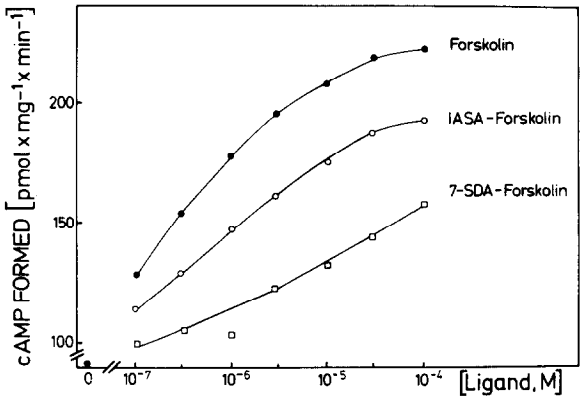

Fig.3. Stimulation of AlF_4^--activated adenylate cyclase from rabbit myocardial membranes. Rabbit myocardial membranes (20 mg/ml, in buffer B) were incubated with 10 mM NaF, 10 μM $AlCl_3$, 6 mM $MgCl_2$ for 1 h at 0°C and tested for adenylate cyclase activity without washing. Treated membranes (100 μg) were incubated in the presence of the indicated concentrations of forskolin or derivatives and 0.1 mM [α-^{32}P]ATP (10 cpm/pmol), 10 mM creatine phosphate, 20 μg/ml creatine kinase, 5 mM theophylline, 5 mM $MgCl_2$, in 20 mM Mops buffer (pH 7.4) for 20 min at 30°C and cyclase activity measured as described [2]. Vehicle for forskolin and derivatives was ethanol (2% final).

was observed when crude membranes (e.g. myocardial or human platelet membranes) were inspected. Therefore, specific labeling of adenylate cyclase could only be achieved using pure or partially purified preparations. Such an experiment is shown in fig.4A for purified AlF_4^--activated myocardial enzyme. Labeling only of the 150 kDa catalytic moiety and not of the α_s portion of the enzyme was noted. Furthermore, photoincorporation was almost completely abolished by simultaneous addition of excess unlabeled forskolin. In contrast to the glucose carrier (fig.4B) and in agreement with the failure to affect adenylate cyclase activity, the azido label experienced no competition at all with D-glucose or cytochalasin B.

One of the benefits in the use of [^{125}I]IASA-forskolin as an affinity label for forskolin-binding sites lies in its ease of preparation. It requires only the radioiodination of a stable precursor, thus avoiding the recurrent multistep preparation of a

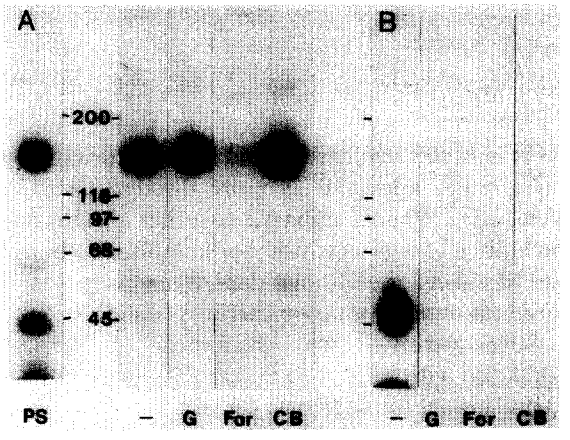

Fig.4. Failure of glucose and cytochalasin B to compete for forskolin-binding sites on adenylate cyclase. (A) Purified $C\alpha_s \cdot AlF_4^-$ complex from rabbit myocardium (0.18 μg = 0.95 pmol) was incubated with 300 nM [^{125}I]IASA-forskolin (200 Ci/mmol in 100 μl buffer B in the absence (–) and presence of 30 μM forskolin (For), 500 mM D-glucose (G) or 10 μM cytochalasin B (CB) for 10 min at 22°C in the dark. PS, protein stain of the same preparation via radioiodination according to Greenwood et al. [20]. (B) Human erythrocyte membranes (0.3 mg/ml) in buffer A were pretreated without (–) and with 30 μM forskolin (For), or 500 mM glucose (G) or 10 μM cytochalasin B (CB) and 5 nM [^{125}I]IASA-forskolin. Further treatment was as described in the legend to fig.1. Samples from (A,B) were irradiated and analysed by SDS gel electrophoresis as described in section 2. Molecular masses for standard proteins are indicated in kDa.

radiolabeled intermediate which must be coupled to the forskolin part of the label [10].

The new affinity label also allows for labeling of the glucose transporters in various tissues where these proteins occur in much lower concentrations as compared to human erythrocyte membranes. In the case of the glucose carrier, the higher affinity of the label with its rather hydrophobic side chain as compared to forskolin is remarkable and confirms the observations made by Wadzinski et al. [10] on a related compound. A similar phenomenon has been reported by us recently [21,22] with GTP analogs. When looking at stimulation of adenylate cyclase via G_s, γ-substituted aryl esters of aryl amidates were shown to be much more efficient than short-chain γ-alkyl esters or the γ-fluoridate of GTP.

Unfortunately, the 'hydrophobic' effect (i.e. gain in affinity) displayed by the affinity label did not apply for the forskolin-binding site of the catalytic subunit of adenylate cyclase. However, the label can be used to tag specifically this site using more purified preparations of the enzyme (fig.4A). Sequence information is available for glucose carriers [23,24] and will certainly be available in the near future for adenylate cyclase as well. It will be of interest to determine how the respective binding sites may differ between these different forskolin-binding proteins, exhibiting such diverse functions.

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