Volume 227, number 2, 225-229 FEB 05503 January 1988

Interactions of pure $\beta\gamma$-subunits of G-proteins with purified β_1-adrenoceptor

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Received 28 October 1987; revised version received 2 December 1987

The role of the $\beta\gamma$-subunits in the interaction of G-proteins was examined with β_1-adrenoceptors purified from turkey erythrocytes and pure $\beta\gamma$-subunits prepared from turkey erythrocytes and bovine brain. On a non-denaturing polyacrylamide gel, the mobility of $\beta\gamma$-subunits was increased when incubated with β_1-adrenoceptor and the β_1-adrenergic agonist l-(−)-isoproterenol, whereas on incubation with the antagonist l-alprenolol the mobility was unchanged. Furthermore, the β_1-adrenoceptor was retarded on a Sephadex G-50 column equilibrated with $\beta\gamma$-subunits and agonist. No retardation occurred in the presence of antagonist. These data suggest a direct interaction of activated β_1-adrenoceptors with isolated $\beta\gamma$-subunits of G-proteins.

β_1-Adrenoceptor; G-protein; $\beta\gamma$-subunit; Protein-protein interaction

1. INTRODUCTION

A family of GTP-binding proteins function as transducers in signal transfer from hormone receptors to adenylyl cyclase [1] and other targets [2]. These G-proteins are all heterotrimers with α-subunits of 39 to 45 kDa which bind GTP and are substrates for toxins, such as cholera toxin and/or pertussis toxin, and β- and γ-subunits which do not bind GTP. The β-subunits have molecular masses of 35 or 36 kDa depending on the source of G-protein [3,4], whereas the γ-subunits are more heterogeneous with molecular masses ranging from 5 to 10 kDa [5]. Only recently, detailed studies were carried out with separate and pure components in reconstituted lipid vesicles to clarify the role of the $\beta\gamma$-subunits in signal transmission from β-adrenoceptors to adenylyl cyclase [6–8]. The results may be summarized as follows: the $\beta\gamma$-subunits are obligatorily required for ADP-ribosylation of G_o, G_i and G_n which are all pertussis toxin substrates [9]. The $\beta\gamma$-subunits stimulate the Mg^{2+}-dependent binding of GTPγS to G_o, G_i and G_n. Addition of a molar excess of $\beta\gamma$-subunits promotes deactivation of $(AlF_4)^-$-activated G_s [10], but most significant is an attenuation of basal, e.g. GTP or GTPγS (non-hormonally) activated adenylyl cyclase by $\beta\gamma$-subunits [6,11]. An anchor role was also ascribed to the γ-subunit for insertion of G-proteins into the membrane [12] and recently reported evidence points to a role of $\beta\gamma$-subunits in receptor G-coupling. For example, a moderate molar excess of $\beta\gamma$-subunits promotes receptor-activated GTPase activity in the case of β-adrenoceptor and G_s [7]

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Abbreviations: β_1AR, β_1-adrenoceptor; G-proteins, guanine nucleotide-binding proteins; G_s, G_i, stimulatory and inhibitory guanine nucleotide-binding proteins of adenylyl cyclase, respectively; G_o, guanine nucleotide-binding protein first found in brain; G_n, guanine nucleotide-binding protein purified from bovine neutrophils; G_t, transducin, a guanine nucleotide-binding protein purified from rod outer segments; GTPγS, guanosine-5′-*O*-3-thiophosphate; GTP, guanosine-5′-triphosphate; DTT, 1,4-dithiothreitol; [^{3}H]DHA, [^{3}H]dihydroalprenolol; cmc, critical micelle concentration; PAGE, polyacrylamide gel electrophoresis

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00145793/88/$3.50

and bleached rhodopsin and $G_{t\alpha}$ or $G_{i\alpha}$ [13,14]. Moreover, $\beta\gamma$-subunits shift partially purified muscarinic acetylcholine receptor co-reconstituted with G_o into lipid vesicles towards the high-affinity state for agonists [15]. A putative direct interaction between $\beta\gamma$-subunits and rhodopsin may be deduced from the fact that a polyclonal antibody preparation against $\beta\gamma$-transducin contains anti-idiotypic antibodies against rhodopsin, because this could be interpreted to mean that rhodopsin has a specific binding domain for $\beta\gamma$-subunits [16]. This interpretation is supported by the fact that transducin $\beta\gamma$-subunits inhibit the phosphorylation of rhodopsin by rhodopsin kinase and interfere with binding of antibodies directed against carboxyl-terminal peptides containing the phosphorylation sites in rhodopsin [17]. To these data suggesting direct interaction between $\beta\gamma$-subunits and receptors we now add information indicating that pure $\beta\gamma$-subunits of G-proteins prepared from turkey erythrocytes and bovine brain interact with β_1-adrenoceptor also purified from turkey erythrocytes when the receptor is activated by a hormone agonist.

2. MATERIALS AND METHODS

The β_1-adrenoceptors from turkey erythrocyte membranes were purified according to published procedures [18,19]. G_s and $G_{i\alpha}$ from turkey erythrocytes were purified as described by Northup et al. [10]. The $\beta\gamma$-subunits of G-proteins were purified to homogeneity either from bovine brain [20] or from turkey erythrocytes [10]. The purified $\beta\gamma$-subunits were concentrated on a hydroxyapatite column and stored at −80°C in 25 mM Hepes buffer (pH 8.0) containing 100 mM NaCl, 1 mM DTT, 0.05% Lubrol-PX and 10% glycerol. The β_1 AR and $\beta\gamma$-preparations were free of $G_{i\alpha}$, $G_{o\alpha}$ and $G_{s\alpha}$ as shown by [^{35}S]GTPγS binding, silver staining [21], [^{32}P]ADP-ribosylation and immunoblotting techniques (see fig.1). [^{32}P]NAD was synthesized as described by Cassel and Pfeuffer [22]. Pertussis toxin catalyzed ADP-ribosylation was carried out by the procedure of Bokoch et al. [23]. Immunoblotting was performed according to Towbin et al. [24] using polyclonal antibodies against $G_{s\alpha}$-peptide (379–394) from bovine brain [25]. The concentrations of β_1-adrenoceptor and $\beta\gamma$-subunits were measured by [^{3}H]DHA binding and the [^{3}H]fluorodinitrobenzene method, respectively [19]. The binding of $\beta\gamma$-subunits to the β_1-adrenoceptor was examined by gel permeation [26,27]. For that purpose, the β_1-adrenoceptor was applied to a Sephadex G-50 column equilibrated with ^{125}I-$\beta\gamma$-subunits (3000 cpm/pmol) in 20 mM Hepes, 100 mM NaCl, 0.5 mM DTT and 0.25% monolauroyl sucrose buffer (pH 8.0). The radioactivity of each fraction was counted.

The similar molecular mass of β_1AR from turkey erythrocytes (40 and 50 kDa) and of $\beta\gamma$-subunit complexes (45 kDa) and comparable isoelectric points (p*I*) 5.8–6.2 [28,29] made it seem attractive to use native polyacrylamide gel in order to detect association between β_1AR and $\beta\gamma$-subunits in the presence of agonist. Non-denaturing polyacrylamide gels (4–5%) were prepared by the method of Hames [30] without a stacking gel. Gel electrophoresis was carried out under the conditions of Laemmli [31] except that SDS was omitted and replaced by 0.25% monolauroyl sucrose for the gel preparation and for the running buffer. Monolauroyl sucrose is a neutral detergent with a high cmc which does not denature βAR and G-proteins and prevents aggregation of hydrophobic proteins [19]. Depending on experimental conditions the gels were prepared with or without ligands. The electrophoresis was carried out overnight at 4°C and 8 mA. β_1AR and $\beta\gamma$-subunits were labelled with ^{125}I by the chloramine-T method [32].

3. RESULTS AND DISCUSSION

The data in fig.2 show an increased mobility of ^{125}I-labelled $\beta\gamma$-subunits in 4% non-denaturing polyacrylamide gels as a function of increasing β_1AR concentrations. The mobility increase was only observed in the presence of 10^{-4} M 1-(−)-isoproterenol in the preincubation and in the gel and in the running buffer. In the control (fig.2B) isoproterenol was replaced in the preincubation and in the gel by 10^{-6} M l-alprenolol. No change of $\beta\gamma$-subunit mobility was recorded. The same experiment was carried out using bovine brain $\beta\gamma$-

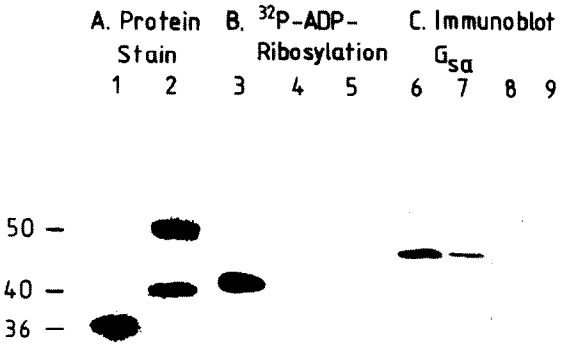

Fig.1. Purity of the components. (A) Silver stain of $\beta\gamma$-subunits from bovine brain (1) and β_1AR from turkey erythrocytes (2) after SDS-PAGE. (B) Autoradiogram of $G_{i\alpha}$ ADP-ribosylated by pertussis toxin. 10 ng $G_{i\alpha}$ (3) from turkey erythrocytes in the presence of 20 ng $\beta\gamma$-subunits, 300 ng $\beta\gamma$-subunits (4) and 300 ng β_1AR (5) in the presence of 20 ng $\beta\gamma$-subunits. (C) Immunoblots of 12.5 ng rabbit liver G_s (6), 2.5 ng G_s (7), 875 ng $\beta\gamma$-subunits (8) and 300 ng β_1AR (9). Blots were processed with rabbit anti-$G_{s\alpha}$-peptide serum (1:750) and with peroxidase-labelled goat antibodies to rabbit IgG. $\beta\gamma$-Subunits from turkey erythrocytes were likewise free from $G_{s\alpha}$ and $G_{i\alpha}$ as estimated by the same techniques (not shown).

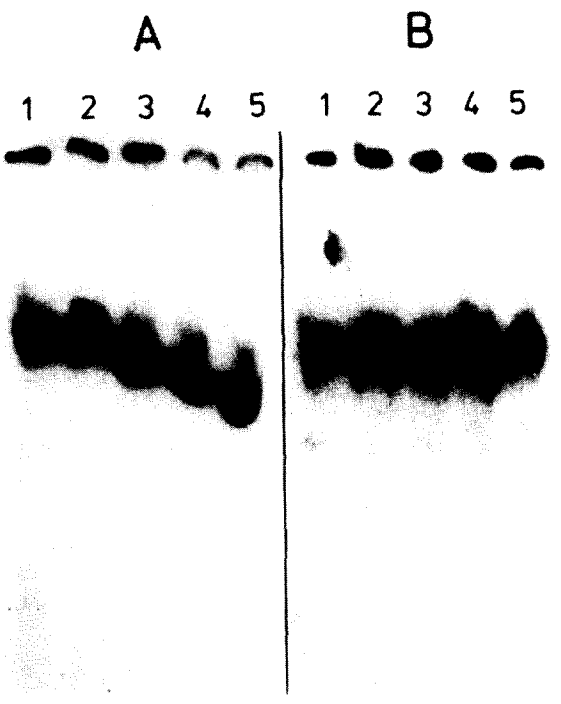

Fig.2. Interactions in non-denaturing polyacrylamide gels between β_1AR and $\beta\gamma$-subunits of G-proteins in the presence of agonist or antagonist. The gels (4%) containing 0.25% monolauroyl sucrose were prepared without stacking gel in the presence of 10^{-4} M l-(−)-isoproterenol or 10^{-6} M l-alprenolol. The running buffer contained 0.1% monolauroyl sucrose and the same concentration of ligands. The samples in A and B contained β_1AR and $\beta\gamma$-subunits with 1500 cpm of ^{125}I-labelled $\beta\gamma$-subunits from turkey erythrocytes and in A 10^{-4} M isoproterenol and in B 10^{-6} M alprenolol in 20 mM Hepes, 0.3 mM DTT, 100 mM NaCl buffer (pH 7.5). The detergent concentrations of digitonin, Lubrol-PX and monolauroyl sucrose in the samples were 0.05%, 0.05% and 0.25%, respectively. The samples were preincubated at 30°C for 30 min. Electrophoresis was carried out overnight at 4°C and 8 mA. (A) Mobilities of $\beta\gamma$-subunits (4 pmol) in the presence of agonist but no addition of β_1AR (lane 1), with 2 pmol β_1AR (lane 2), with 3 pmol β_1AR (lane 3), with 4 pmol β_1AR (lane 4) and with 5 pmol β_1AR (lane 5). (B) Mobilities of $\beta\gamma$-subunits (5 pmol) in the presence of antagonist but no addition of β_1AR (lane 1), with 2 pmol β_1AR (lane 2), with 4 pmol β_1AR (lane 3), with 6 pmol β_1AR (lane 4) and with 8 pmol β_1AR (lane 5).

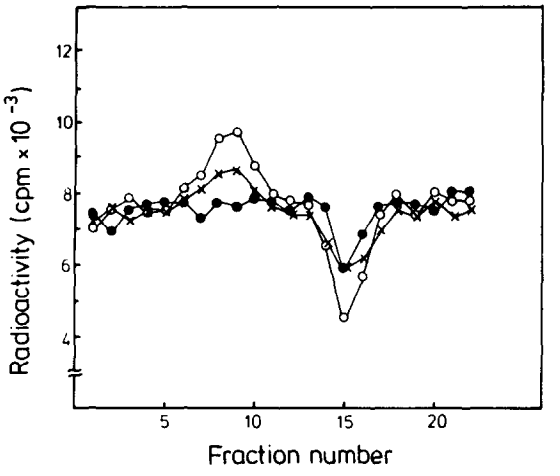

Fig.3. Elution profiles of purified β_1-adrenoceptor from a Sephadex G-50 column equilibrated with ^{125}I-labelled $\beta\gamma$-subunits. A Sephadex G-50 column (0.4 × 8.0 cm) was equilibrated with 20 mM Hepes, 100 mM NaCl, 0.5 mM DTT and 0.25% monolauroyl sucrose buffer (pH 8.0) which contained 30 nM ^{125}I-$\beta\gamma$-subunits (3000 cpm/pmol) from bovine brain with 10^{-4} M l-(−)-isoproterenol (○—○), or with 10^{-6} M l-alprenolol (•—•) and without ligands (×—×). The column equilibration was carried out at room temperature. The β_1-adrenoceptor (2 pmol) from turkey erythrocytes was preincubated with or without ligand for 15 min at room temperature and then applied to the column (flow rate = 70 μl/min). Each fraction (100 μl) was counted for radioactivity. The data are representative for three identically performed experiments.

subunits (see fig.4A). The results were like those obtained with the $\beta\gamma$-subunits from avian erythrocytes. The converse experiments were also carried out using ^{125}I-βAR and increasing concentrations of $\beta\gamma$-subunits. Similar results like those shown in fig.2A were obtained. The mobility of β_1AR in the presence of $\beta\gamma$-subunits was faster than in their absence. Again the mobility increase was only seen in the presence of isoproterenol (not shown). Binding of $\beta\gamma$-subunits to β_1AR was also followed by equilibrium binding on a gel-filtration column (fig.3). A peak of ^{125}I-radioactivity corresponding to a $\beta\gamma$-receptor complex was observed in the presence of isoproterenol. Assuming a 1:1 complex of $\beta\gamma$ and β_1AR, the radioactive peak is consistent with 1.78 pmol of 2 pmol β_1AR applied. On the other hand, the antagonist prevented the interaction of β_1AR with $\beta\gamma$-subunits like in the above experiments. But a smaller peak of ^{125}I-$\beta\gamma$-subunits was observed even in the absence of ligand indicating an interaction between non-liganded βAR and $\beta\gamma$. A similar, but considerably smaller trough centered at fraction 15 in the presence of alprenolol results from the fact that the receptor was applied to the column in the absence of $\beta\gamma$-subunits. In order to explain the clear separation of the $\beta\gamma$-adrenoceptor complex, additional absorptive forces exerted by the Sephadex

matrix must be postulated. It was noted that among the conditions for preincubation, temperature was crucial: when the temperature for the preincubation was lowered from 30°C to 4°C the mobility change in the presence of agonist was not observed (cf. fig.4A and B).

A considerable effort was made to detect a change in the affinity of the β_1AR on co-reconstitution with $\beta\gamma$-subunits into lipid vesicles using methods established in this laboratory [7,18]. However, the reconstituted turkey β_1AR did not respond well to interaction with either non-activated or activated forms of G_s with a shift from a high-affinity to low-affinity state in the presence of the hormone agonist. But, one should remember that in turkey membranes the sensitivity of this shift is controversial [33–35]. This unfavorable property of the turkey β_1AR might have been responsible for the failure to obtain unequivocal effects with co-reconstituted $\beta\gamma$-subunits on ligand displacement at the receptor.

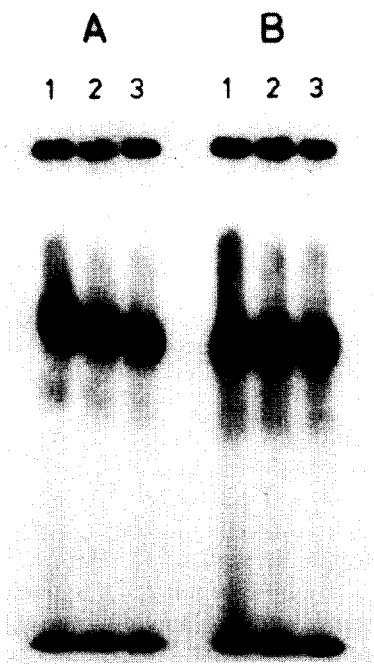

Fig.4. Influence of temperature on the mobility of $\beta\gamma$-subunits. The non-denaturing gel (5%) containing 0.25% monolauroyl sucrose was prepared without ligand, but the running buffer contained 10^{-4} M l-(−)-isoproterenol and 0.3 mM DTT. The samples contained turkey erythrocyte β_1AR and $\beta\gamma$-subunits from bovine brain and 10^{-4} M isoproterenol in 20 mM Hepes, 100 mM NaCl buffer (pH 7.5). The detergent concentrations in the samples were 0.05% digitonin, 0.05% Lubrol-PX and 0.25% monolauroyl sucrose. In A the reaction mixtures were incubated for 30 min at 30°C and in B for 1 h in an ice bath. Electrophoresis was carried out in both cases overnight at 4°C. (A) Mobilities of $\beta\gamma$ (3 pmol) after incubation at 30°C in the presence of agonist but without β_1AR (lane 1), with 2 pmol β_1AR (lane 2) and with 4 pmol β_1AR (lane 3). (B) As in A, but after incubation in an ice bath.

The experiments reported here show that purified turkey β_1AR can interact with $\beta\gamma$-subunits of G-proteins from the same source or from bovine brain. The consequence of this interaction probably is formation of a 'hormone-β_1AR $\beta\gamma$-complex'. The molecular basis of the conformational changes responsible for the βAR-$\beta\gamma$ interaction is not known. The mobility change of the interacting proteins suggests a decreased pK, but for what discrete structural change, protonation-deprotonation might be indicative is as obscure as is a possible functional significance for interactions of β-adrenoceptors with $\beta\gamma$-subunits in the course of signal transmission in the native membrane.

Acknowledgements: Dr D. Palm from this Department kindly supplied us with polyclonal antibodies against $G_{s\alpha}$-peptide from bovine brain (DFG grant Pa 92/20). We are greatly indebted to Miss P. Fischer and Mrs B. Fischer for excellent technical assistance throughout this work. This work was supported by SFB 176 and Fonds der Chemischen Industrie eV.

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