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GTP-binding protein associated with amino acid binding proteins from olfactory epithelium of skate, *Dasyatis pastinaca*

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Amino acid binding protein (98 kDa) from olfactory epithelium of skate, *Dasyatis pastinaca*, form a stable complex with the 56 kDa protein. This complex dissociates in the presence of 10 μM GTPγS and 2 mM $MgCl_2$. The 56 kDa protein has a steady-state GTPase activity (15 nmol/min per mg). Binding of amino acids to the 98 kDa protein specifically stimulates GTPase activity of the 56 kDa protein; half-maximal stimulation of GTPase activity is observed at 0.1 μM amino acid.

Olfaction; Amino acid binding protein; Receptor; G-protein; *(Dasyatis pastinaca)*

1. INTRODUCTION

Recently a number of membrane proteins were isolated from olfactory epithelium of skate, *Dasyatis pastinaca*, using affinity chromatography on agarose with coupled amino acid [1]. They specifically bound amino acid ('alanine-serine', 'glutamate' and 'lysine' binding proteins). A number of properties of the isolated proteins indicated that they could represent the most probable candidates for olfactory receptor molecules in fish. These 'receptor' molecules were isolated as a complex of two polypeptides: 98 and 56 kDa (98/56 kDa complex). 98 kDa polypeptides specifically bound amino acid and, obviously played the main role in odor recognition. The function of the 56 kDa polypeptide remained unknown. In this paper we tried to determine the function of the 56 kDa polypeptide and to investigate its interaction with amino acid binding proteins (98 kDa).

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2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]GTP (>1000 Ci/mmol; USSR), GTP, GTPγS, L-alanine and L-glutamic acid were from Sigma (St. Louis, USA); chemicals for electrophoresis were from Reanal (Hungary).

The synthesis of L-alanine-agarose and L-glutamate-agarose was performed by a standard method [2]. The ligands were coupled to AH-agarose (Pharmacia, Uppsala, Sweden) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The capacity was 3.7 μmol/ml for L-alanine-agarose and 3.4 μmol/ml for L-glutamate-agarose.

IgG-agarose was prepared using CNBr-activated agarose (Pharmacia) and rabbit IgG raised against the 56 kDa protein. The 56 kDa protein from skate olfactory epithelium was isolated earlier [1].

2.2. *Preparation of Triton X-100 extract of olfactory epithelium tissue*

The 20000 × *g* pellet of skate olfactory epithelium was obtained as previously described [1,3]. It was solubilized in 0.1 M Tris-HCl buffer, pH 7.2, containing 0.3% Triton X-100 and 100 μM phenylmethylsulfonyl fluoride (PMSF). Triton X-100 insoluble material was removed by centrifugation at 20000 × *g* for 1 h (Triton X-100 extract).

2.3. *Affinity chromatography on L-alanine- and L-glutamate-agarose*

The columns (0.5 × 2 cm) were packed with L-alanine-agarose ('alanine' column) or L-glutamate-agarose ('glutamate' column). The columns were equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 0.05% Triton X-100 and 0.2 M NaCl. Triton X-100 extract (40 ml volume, 20 mg protein) was

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applied to columns at 4°C. Flow rate was 20 ml/h. The absorbed proteins were eluted with 0.2 M acetate buffer, pH 3.0, at 25°C.

2.4. *Affinity chromatography on IgG-agarose*

The column (0.5 × 2 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 0.05% Triton X-100 and 0.5 M NaCl. Triton X-100 extract (60 ml volume, 30 mg protein) was applied to the column at 4°C. Flow rate was 20 ml/h. The absorbed proteins were eluted with 20 mM glycine-HCl buffer, pH 2.8, containing 0.05% Triton X-100 and 0.5 M NaCl.

After both types of chromatography the isolated proteins were dialyzed against 50 mM Tris-HCl buffer, pH 7.2, containing 0.01% Triton X-100 and 0.1 M NaCl to study the interaction of 98 and 56 kDa proteins, or against 100 mM Hepes-KOH buffer, pH 7.4, containing 2 mM $MgCl_2$ when GTPase activity was determined.

2.5. *GTPase activity*

GTPase activity was determined according to [4]. Protein (2 μg in 100 μl buffer) was incubated with 4 mM GTP and 0.5 μCi [γ-^{32}P]GTP for 10 min.

2.6. *Electrophoresis*

Electrophoresis in 10% polyacrylamide gel was carried out according to Laemmli [5]. The proteins were visualized by Amersham's silver stain kit 'Quick-silver' (Amersham, England).

Protein concentration was determined as in [6].

3. RESULTS AND DISCUSSION

Fig.1a shows SDS-gel electrophoresis patterns of the proteins isolated by alanine and glutamate columns from Triton X-100 extract of skate olfactory epithelium. Two proteins were eluted from the columns of M_r 98000 and M_r 56000. These proteins (98/56 kDa complex) were described earlier and the 98 kDa proteins were able to bind amino acids [1]. Henceforth, the 98 kDa protein isolated from the alanine column will be designated by 98_{Ala}, and that from the glutamate column by 98_{Glu}.

If the amino acid binding 98 kDa proteins are indeed olfactory receptor molecules, it is reasonable to assume that the 56 kDa protein may be involved in signal transduction. For many receptor systems it is known that a receptor forms a stable complex with α-subunits of G-protein when agonist binds to a receptor molecule [7]. The chromatography of any receptors on sorbents with coupled agonist is equal to agonist binding with a receptor. So, during the chromatography on agarose with coupled amino acid a ternary complex may be formed: agonist (amino acid)–receptor (98 kDa)–α-subunit of G-protein (56 kDa).

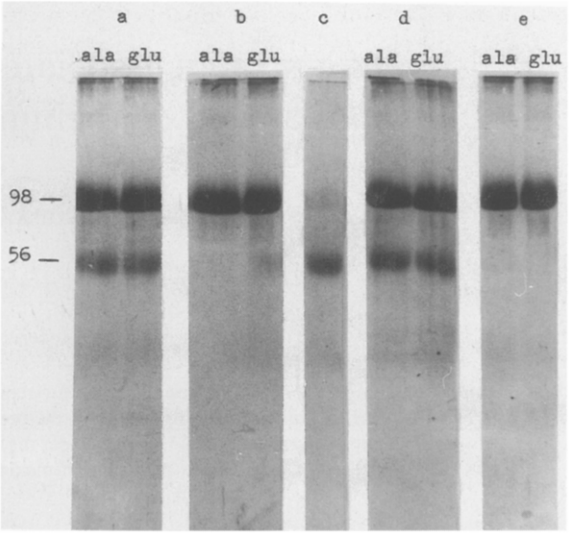

Fig.1. SDS-gel electrophoresis patterns of proteins isolated by affinity chromatography under different conditions. (a) Chromatogram of Triton X-100 extract on alanine (ala) and glutamate (glu) columns; (b) the same in the presence of 10 μM GTPγS and 2 mM $MgCl_2$; (c) chromatogram of Triton X-100 extract on agarose with coupled IgG against 56 kDa protein; (d) rechromatogram of mixture of the separately isolated 98_{Ala}, 98_{Glu} and 56 kDa proteins on alanine (ala) and glutamate (glu) columns; (e) the same in the presence of 10 μM GTPγS and 2 mM $MgCl_2$. Silver staining.

The complex receptor–α-subunit of G-protein dissociates in the presence of GTP or its nonhydrolyzable analogues [7]. So, only the receptor should bind to the affinity column in the presence of GTP, GTPγS, Gpp[NH]p. In our experiments only 98_{Ala} or 98_{Glu} proteins were eluted from alanine or glutamate columns when Triton X-100 extract was chromatographed in the presence of 10 μM GTPγS (fig.1b).

The separately isolated 98 and 56 kDa proteins were able to reassociate. In these experiments 98_{Ala} and 98_{Glu} proteins were isolated on the alanine or glutamate columns in the presence of GTPγS and the 56 kDa protein was isolated from Triton X-100 extract by agarose with coupled rabbit IgG raised against 56 kDa protein (fig.1c). When the mixture of the separately isolated 98_{Ala}, 98_{Glu} and 56 kDa proteins was rechromatographed on alanine or glutamate columns, both proteins (98 and 56 kDa) absorbed on the columns (fig.1d), but only 98_{Ala} or 98_{Glu} proteins absorbed on the columns in the presence of 10 μM GTPγS (fig.1e).

G-proteins have GTPase activity which is

associated with α-subunits. In our experiments only 56 kDa protein had GTPase activity, amounting to 15 ± 4 nM/min per mg which is similar to the specific activity of well-known G-proteins [7].

The GTPase activity revealed remained in the 98/56 kDa complex and considerably increased in the presence of amino acid (table 1). This result is in good agreement with the data suggesting that agonist-receptor complex stimulates the steady-state GTPase activity of G-proteins. Half-maximal stimulation of GTPase activity of the 98/56 kDa complex was observed at 0.1 μM amino acid.

The activation of GTPase activity of the 98/56 kDa complex was specific to amino acid (table 2). The data in table 2 indicate that specificity to an odorant is determined by the 98 kDa protein. The 56 kDa protein appears to be universal and its GTPase activity may be stimulated by any 98 kDa proteins.

So, the following properties of the 56 kDa protein from the olfactory epithelium of skate coincide with those of α-subunits of well-known G-proteins: (i) in the presence of agonist (amino acid) it forms a stable complex with the 98 kDa amino acid binding protein (the most probable olfactory receptor molecule); (ii) this complex dissociates in the presence of GTPγS; (iii) the 56 kDa protein has a steady-state GTPase activity; (iv) binding of amino acid to 98 kDa proteins specifically stimulates GTPase activity of the 56 kDa protein.

The molecular mass of the 56 kDa protein is higher than the molecular mass of α-subunits of most known G-proteins (39–52 kDa [8,9]) including G-proteins found in the olfactory epithelia of various species [10–13]. However, an intensive study of G-proteins has revealed many unknown forms of G-proteins [7]. For example, α-subunit of G-protein associated with PI-phospholipase C has a molecular mass of 54 kDa [14]. On the other hand, amino acids activate PI-phospholipase C in isolated fish olfactory cilia [15]. It is not expected that the 56 kDa protein from skate olfactory epithelium modulates phosphatidylinositol metabolism in olfactory receptor cells of fish.

A number of properties of the 98 kDa protein suggest the 98 kDa protein to be the most probable candidate for olfactory receptor in fish [3]. Activation of the 56 kDa protein GTPase activity by the amino acid-98 kDa protein complex is a strong argument for this. Recently odor binding glycoproteins with receptor properties were isolated from rat olfactory epithelium. These glycoproteins were isolated as a complex consisting of two subunits: the 88 kDa odor binding subunit and the 55 kDa subunit [16,17]. It is not expected that these proteins constitute an olfactory receptor molecule (88 kDa) and an α-subunit of G-protein (55 kDa). This is supported by the preliminary data of cross-immunoreactivity between the 55 kDa protein from rat and the 56 kDa protein from skate which revealed the presence of common determinants for both proteins.

Table 1

Alanine-stimulated activation of GTPase activity of 98/56 kDa complex isolated on alanine column[a]

L-Alanine added (μM)	GTPase activity
0.00	1.0
0.01	1.1
0.03	1.75
0.10	1.9
0.30	3.3
1.00	2.6

[a] GTPase activity was taken as 1.0 in the absence of L-alanine

Values are averages of triplicate determinations from a representative experiment

Table 2

GTPase activity of the isolated proteins from skate olfactory epithelium[a]

Protein	Stimulus	GTPase activity
98_{Ala} + 98_{Glu}	–	n.d.
56 kDa	–	1.0
56 kDa	Ala + Glu	1.0
56 kDa + 98_{Ala}	–	0.75
56 kDa + 98_{Ala}	Ala	1.85
56 kDa + 98_{Ala}	Glu	0.8
56 kDa + 98_{Glu}	–	0.85
56 kDa + 98_{Glu}	Ala	0.95
56 kDa + 98_{Glu}	Glu	2.20

[a] The amount of each indicated protein in the sample is 2 μg. Concentration of amino acid in the sample is 1 μM. GTPase activity of 56 kDa protein in the absence of amino acid was taken as 1.0

Values are averages of triplicate determinations from a representative experiment

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