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Monoclonal antibodies against the S_2-serotonin receptor from rat brain that cross-react with dopamine and opiate receptors

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Balb/c mice were immunized with rat striatal integral membrane proteins. After hybridization of splenocytes with myeloma cells, hybridoma lines secreting antibodies against serotonin, dopamine and opiate receptors were detected by inhibition of ligand binding to brain membrane preparations. Antibodies from two positive lines, Mab/a9 and Mab/a18, were able to inhibit ligand binding to the S_2-serotonin (K_d range: 10–100 nM), the μ-opiate (K_d range: 0.4–3 μM) and the δ-opiate receptors (K_d range: 0.7–1.1 μM), while Mab/a9 was also found to inhibit ligand binding to the D_2/D_4-dopamine receptor ($K_d \sim 50$ nM). An apparent molecular mass of 60 kDa could be ascribed to the δ-opiate receptor and apparent molecular masses of 29 and 36 kDa to the μ-opiate receptor by ligand elution from immuno-precipitates.

Monoclonal antibody; Serotonin receptor; Dopamine receptor; Opiate receptor

1. INTRODUCTION

In vitro binding studies and in vivo pharmacokinetics with various neurotransmitter agonists or antagonists [1–3] have indicated that, in general, neurotransmitters and their analogues are not strictly specific to a certain receptor. For example, serotonin acts on the S_1-serotonin receptors in nanomolar, but on the S_2-serotonin receptors in micromolar concentrations, while spiperone shows nanomolar affinities for both the S_2-serotonin and D_2/D_4-dopamine receptors [2,3]. Similarly, opioids like etorphine and ketacocine recognize δ-, μ- and $\varkappa$-opiate receptors with comparable affinities [4]. The opiate drug fentanyl binds to opiate receptors at nanomolar concentrations and to dopamine receptors at micromolar concentrations [3]. This range of specificities implies that similar structural domains presumably prevail in the ligand-binding sites of neurotransmitter receptors.

In the following, we present a study where monoclonal antibodies which were initially directed against the binding sites of the δ- and μ-opiate receptors were found to cross-react with S_2-serotonin and D_2/D_4-dopamine receptors.

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Abbreviations: Tris buffer, 50 mM Tris adjusted with HCl to pH 7.4; DMEM, Dulbecco's modified Eagle's medium

2. MATERIALS AND METHODS

2.1. *Membrane preparation*

Crude synaptosomal membranes (P_2m) were prepared from SPD rat brains or neuroblastoma × glioma NG108-15 cells [5]. Final membrane preparations were suspended in 50 mM Tris-HCl buffer, pH 7.4 (Tris buffer) at 2 mg protein/ml. For immunization (see below) P_2m membranes from rat striatum were first treated with 0.1 M NaOH for removal of peripheral proteins [6]. 1 vol. pelleted membranes was resuspended in 9 vols ice-cold 0.1 M NaOH and immediately centrifuged at 25000 × g (10 min, 4°C). The resulting pellet was then washed 3 times with Tris buffer at 4°C. About 90% of the membrane proteins were detached by this procedure and virtually no tubulin, actin or spectrin-like proteins remained in the membrane as indicated by SDS-polyacrylamide gradient gel electrophoresis (gradient: 7.5–15%). The remaining membrane proteins were further fractionated according to molecular mass by preparative SDS-polyacrylamide gel electrophoresis with an intermediate agarose layer [7] as follows. The gel (approx. 15 cm) was divided into three layers (from bottom to top): (i) 2 cm of 20%

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polyacrylamide, (ii) 2–3 cm of 1% agarose (in Tris buffer) and (iii) 5 cm of 6% polyacrylamide. Membrane proteins (2–5 mg) were dissolved in reducing sample buffer and loaded over the whole stacking gel with the exception of one slot, which was reserved for the molecular mass markers (Sigma) labelled with fluorescamine (Sigma). Electrophoresis was carried out until fluorescamine-labelled carbonic anhydrase (29 kDa) entered the agarose layer. Proteins were recovered from the agarose by ultracentrifugation and the remaining 6% polyacrylamide gel was cut into two strips above fluorescamine-labelled bovine serum albumin. These strips were kept frozen at −20°C until use. Elution of the proteins in the remaining strips was carried out by a further gel electrophoresis. This gel consisted of the following layers (from bottom to top): (i) 2 cm of 20% polyacrylamide, (ii) 2–3 cm of 1% agarose, (iii) 6% polyacrylamide strip (upside down, i.e. higher molecular mass down), (iv) 2–3 cm of 1% agarose, and (v) 6% polyacrylamide strip. Electrophoresis was carried out until the fluorescent markers passed the 6% polyacrylamide strips and elution from the 1% agarose was carried out as above. Three fractions were obtained by this procedure: fraction I, <30 kDa; fraction II, 30–66 kDa and fraction III, 66–300 kDa. In this study only fraction II was used for immunization.

2.2. *Immunization*

Balb/c mice (female; age, 12 weeks) were injected with 2 × 50 μl containing the sample (150 μg protein) in complete Freund's adjuvant. Identical injections were given in the front footpads 14 days later. After 6 weeks animals were injected intraperitoneally on the sixth, fifth and fourth day before fusion with 200 μg protein dissolved in 100 μl Tris buffer [8].

2.3. *Fusion*

Spleen cells (10^8) obtained from two immunized mice, and 2×10^7 NSO/1 mouse myeloma cells were suspended in 3 ml DMEM (Dulbecco's modified Eagle's medium, supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 500 U penicillin/ml and 1 μg fungizone/ml). Fusion was then induced by polyethylene glycol 1450 (Sigma), as described [8]. After resting for 2 days in DMEM supplemented with 15% fetal calf serum, cells were diluted to a final concentration of 5×10^5 cells/ml containing 1% agar (Difco), hypoxanthine (0.2 μM), aminopterin (1 nM) and thymidine (10 nM) and seeded on agar plates. About 1000 clones were transferred into Costar plates and screened for activity (see below). The positive hybridoma cell lines were propagated as either ascites or in vitro cell cultures [8].

2.4. *Purification of monoclonal antibodies*

Monoclonal antibodies from cell cultures or ascites were purified by anti-mouse antibody affinity chromatography (Sigma). 0.1 M glycine-HCl, pH 2.7, was used as an elution buffer [8]. The isolated antibodies were then dialyzed against the appropriate buffer after neutralization.

2.5. *Screening assay*

Decrease in total ligand binding to a specific neurotransmitter receptor was used as an indication for the presence of anti-receptor antibodies in the hybridoma supernatants. The following radioactive ligands were used: [^{3}H]morphine (79.2 Ci/mol, NEN) for the μ-opiate receptor [4], [^{3}H](D-Ala-Met)-enkephalinamide (39.4 Ci/mol, NEN) for the δ-opiate receptor [4], and [^{3}H]spiperone (31.7 Ci/mol, NEN) for the S_2-serotonin and D_2/D_4-dopamine receptors [2,3,10]. The assays were carried out as follows: Membranes (P_2m) from rat striatum (300 μg protein in 100 μl Tris buffer) were incubated with 300 μl supernatant from hybridoma cultures for 1 h at 25°C. Samples were then cooled to 4°C, supplemented with 300 μl ice-cold Tris buffer and centrifuged for 10 min (2000 × g, 4°C). Pellets were resuspended in 300 μl Tris buffer supplemented with 1 mM EDTA and transferred to microtiter plates (Falcon 4075). Tris buffer (50 μl) containing the radioactive ligand was added to a final concentration of 10 nM for [^{3}H](D-Ala^2-Met^5)-enkephalinamide, and 5 nM for [^{3}H]morphine and [^{3}H]spiperone. Bound ligand was separated from free by filtration (Scatron cell harvester) through Whatman glass fiber filter (934-AH) after 8 min incubation at 25°C. Membranes were then washed for 5 s with ice-cold Tris buffer. After drying, 3 ml Lumax was added to scintillation vials containing the filters. Radioactivity was measured after 12 h.

2.6. *Receptor assay*

Binding assays were performed according to [2,3,9,10]. Protein concentration was determined colorimetrically [11]. The concentrations used for inhibition studies, were 3 nM [^{3}H](D-Ala^2-Met^5)-enkephalinamide, 1 nM [^{3}H]dihydromorphine (90.0 Ci/mmol, NEN) and [^{3}H]spiperone (31.7 Ci/mmol, NEN). Non-specific binding was determined using 1 μM levallorphan for the δ- and μ-opiate receptors, 1 μM mianserin for S_2-serotonin receptors and 1 μM haloperidol for D_2/D_4-dopamine receptors.

2.7. *Immuno-precipitation*

Membranes (~10 mg P_2m from whole rat brain minus cerebellum) were solubilized with 5 ml of 10 mM Chaps/Tris buffer, pH 7.4 [12]. After ultracentrifugation (1 h, 120000 × g, 4°C), aliquots of 1 ml supernatant were incubated in 1.5-ml Eppendorf tubes with 0.7–1 μM monoclonal antibodies (Mab/a9 or Mab/a18) for 30 min at room temperature and an additional 30 min in the presence of 40 μl insoluble protein A (Sigma). The receptor-monoclonal antibody-protein A complex was washed 4 times with ice-cold 2 mM Chaps/Tris buffer, pH 7.4. Elution of bound receptors was carried out with 1 nM (D-Ala^2-Leu^5)-enkephalinamide (Sigma) for the δ-opiate receptor [10] or 1 nM (D-Ala^2,N-Me-Phe^4,Met(O)^5-ol)-enkephalin for the μ-opiate receptor [10].

2.8. *Radio-iodination of putative opiate receptors*

Proteins, obtained by immuno-precipitation, were iodinated with ^{125}I by the chloramine T method [13] and separated from free ^{125}I by size-exclusion chromatography (Sepharose G-25 fine, Pharmacia) using 0.01% SDS in H_2O as eluant. After SDS-polyacrylamide gradient (7.5–15%) gel electrophoresis [14], radioactive proteins were visualized by autoradiography (Agfa-Gevaert, Curix RP2 X-ray film).

3. RESULTS

Striatal rat membranes, enriched with integral proteins, were prepared by mild alkaline treatment to remove practically all peripheral proteins [6].

About 90% of the protein content was removed by this procedure. Fractionation by preparative gel electrophoresis resulted in an additional 3-fold enrichment. Fraction II (M_r 30000–66000), which presumably contained the δ- and μ-opiate receptors [15–17], was then used for immunization. About 1000 hybridoma clones were then produced and their antibodies were screened for decrease in total receptor-ligand binding. The employed ligands were [^{3}H](D-Ala-Met)-enkephalinamide for the δ-opiate receptor, [^{3}H]morphine for the μ-opiate receptor and [^{3}H]spiperone for S_2-serotonin and D_2/D_4-dopamine receptors.

Since optimal binding of these ligands requires special media (e.g. absence of Na^+ for binding of opiate agonists), it was not possible to incubate simultaneously ligands and the supernatants of the hybridoma cultures with membrane receptor preparations. The assay, therefore, consisted of first reacting the membranes with the antibody, followed by exchanging the incubation media (appropriate for ligand binding) and immediate incubation with the radioactive ligand. Bound antibody continuously dissociates under these conditions. We estimated that antibodies with dissociation constants lower than 5 μM could be detected by inhibition of ligand binding within approx. 10 min of the second incubation. In order to achieve such a high rate of ligand association, membrane receptor concentrations as well as ligand concentrations were relatively high (see section 2.5) and the binding assay was performed at 25°C instead of 4°C. Under such conditions saturation isotherms were reached after 8 min incubation (not shown). The timing of the second incubation (8 min) was a critical parameter and, therefore, accurately maintained.

Employing this screening procedure, several hybridoma lines were found to secrete antibodies which inhibit serotonin, dopamine and opiate receptor binding. Monoclonal antibodies from two clones, Mab/a9 and Mab/a18, were studied in more detail. Mab/a9 and Mab/a18 could inhibit ligand binding to the S_2-serotonin, as well as to the δ- and μ-opiate receptor (figs 1,2) at nanomolar and micromolar concentrations, respectively (table 1). Mab/18 did not inhibit D_2/D_4-dopamine binding, while Mab/a9 was able to displace [^{3}H]spiperone binding with an apparent K_d of about 50 nM (fig.1, table 1). Other ^{3}H-labelled

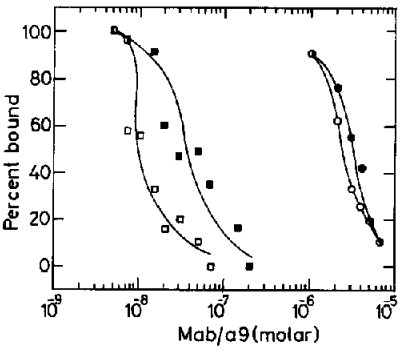

Fig.1. Inhibition of receptor ligand binding by Mab/a9. P_2m membranes from whole rat brain minus cerebellum were first incubated with increasing amounts of Mab/a9 antibodies and then with either 3 nM [^{3}H](D-Ala-Met)-enkephalinamide (●), 1 nM [^{3}H]dihydromorphine (○), a mixture of 1 nM [^{3}H]spiperone and 1 μM mianserin (□) or a mixture of 1 nM [^{3}H]spiperone and 1 μM haloperidol (■) as described in section 2.5.

ligands, like [^{3}H]naloxone (opiate antagonist) [4], ^{3}H]dipremorphine ($\varkappa$-opiate agonist) [18] and [^{3}H]serotonin (S_1-serotonin agonist) [2], could not be displaced by Mab/a9 or Mab/a18 up to final antibody concentrations of 10 μM (table 1).

Inhibition curves of [^{3}H](D-Ala-Met)-enkephalinamide and [^{3}H]dihydromorphine binding (figs 1,2) were used to define the appropriate concentration of monoclonal antibodies for subsequent analysis of the mode of inhibition (i.e. competitive or non-competitive) according to Scatchard [19].

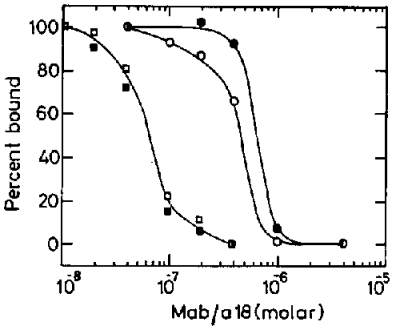

Fig.2. Inhibition of receptor-ligand binding by Mab/a18. P_2m membranes from whole rat brain minus cerebellum were first incubated with increasing amounts of Mab/a18 antibodies and then with either 3 nM [^{3}H](D-Ala-Met)-enkephalinamide (●), 1 nM [^{3}H]dihydromorphine (○), a mixture of 1 nM [^{3}H]spiperone and 1 μM mianserin (■), or 1 nM [^{3}H]spiperone (□).

Table 1

Binding characteristics of monoclonal antibodies Mab/a9 and Mab/a18

	Opiate receptors					Serotonin and dopamine receptors	
	δ		μ		$\varkappa$		
	K_d (μM)	Mode	K_d (μM)	Mode		S_2-type K_d (nM)	D_2/D_4 K_d (nM)
Mab/a9	0.7	c.	2.9	nc.	nb.	~10	~50
Mab/a18	1.1	nc.	0.4	c.	nb.	~60	nb.

K_d, apparent dissociation constant; c., competitive binding; nc., non-competitive binding; nb., no binding (K_d > 10 μM)

The results, presented in figs 3 and 4 and summarized in table 1, indicate that Mab/a9 binds non-competitively to the μ-opiate receptor with an apparent K_d of 2.9 μM and competitively to the δ-opiate receptor with an apparent K_d of 0.7 μM.

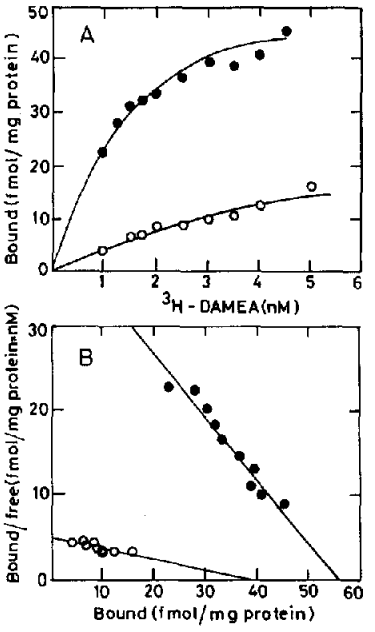

Fig.3. Mode of Mab/a9 binding to the δ-opiate receptor. (A) Binding curve of [^{3}H](D-Ala-Met)-enkephalinamide ([^{3}H]-DAMEA). P_2m membranes from whole rat brain minus cerebellum and increasing amounts of [^{3}H](D-Ala-Met)-enkephalinamide were incubated with (○) and without (●) 3 μM Mab/a9. (B) Scatchard analysis of the above data.

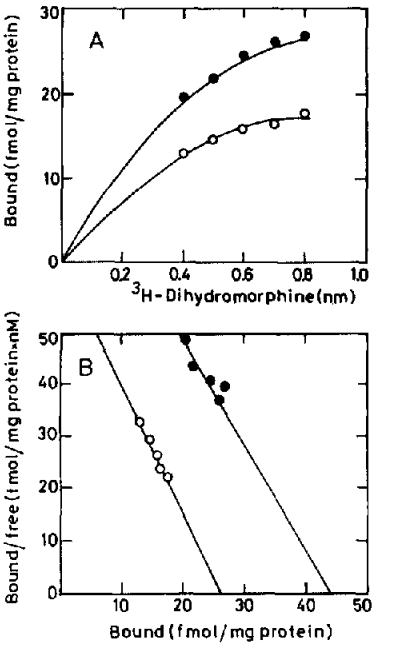

Fig.4. Mode of Mab/a9 binding to the μ-opiate receptor. (A) Binding curve of [^{3}H]dihydromorphine. P_2m membranes from whole rat brain minus cerebellum and increasing amounts of [^{3}H]dihydromorphine were incubated with (○) and without (●) 2 μM Mab/a9. (B) Scatchard analysis of the above data.

Conversely, Mab/a18 binds competitively to the μ-opiate receptor with an apparent K_d of 0.4 μM and non-competitively to the δ-opiate receptor with an apparent K_d of 1.1 μM.

The monoclonal antibodies Mab/a9 and Mab/a18 could provide a means for the isolation and characterization of the S_2-serotonin and D_2/D_4-dopamine receptors as well as the δ- and μ-opiate receptors. Among these receptors, only the δ- and the μ-opiate receptors have been characterized thus far with respect to their molecular mass [15–17]. It was, therefore, decided to concentrate in this study on the opiate receptor. Opiate receptors were solubilized in an active form and immunoprecipitated (see section 2.7). The δ-opiate agonist (D-Ala^2-Leu^5)-enkephalinamide and μ-opiate agonist (D-Ala^2,N-Met-Phe^4,Met(O)^5-ol)-enkephalin were then used to dissociate the respective receptor from the immuno-precipitate with Mab/a18. The proteins in the supernatant were radio-iodinated and analyzed by SDS gel electrophoresis (see section 2.8). The autoradiograms revealed three bands of apparent molecular masses

60, 39 and 29 kDa with either one of the opiate agonists (not shown). In an analogous experiment, a single protein band with an apparent molecular mass of 60 kDa was identified with membranes of neuroblastoma × glioma (NG108-15) cells. Since these cells are known to contain δ-opiate, but not μ-opiate receptors [16], this protein band could be attributed to the δ-opiate receptor in agreement with the results of Simonds et al. [15,16], who obtained a value of 58 kDa for the molecular mass of this receptor by affinity labelling. The remaining species (36 and 29 kDa) might be tentatively attributed to the μ-opiate receptor [17].

4. DISCUSSION

Despite the great demand for monoclonal antibodies against membrane receptors, their production has been successful in only a few cases [20,21]. The major obstacle in the preparation of monoclonal antibodies for the dopamine, serotonin or opiate receptors is undoubtedly their minute amount in biological tissues. Here, instead of using purified receptors, we have used enriched receptor preparations for immunizations. The screening method for positive clones was based on inhibition of receptor ligand binding. The monoclonal antibodies of 2 positive clones, Mab/a9 and Mab/a18, were studied in detail and their basic characteristics are listed in table 1. Both antibodies were found to display high affinities for the S_2-serotonin receptor and low affinities for the δ- and μ-opiate receptors. Mab/a9 was also found to inhibit ligand binding to the D_2/D_4-dopamine and S_2-serotonin receptors with similar affinities (figs 1,2).

Since data on molecular masses for the δ- and μ-opiate receptors are now available [15–17], we have investigated the purification potential of our monoclonal antibodies with these receptors. Three protein bands of 60, 36 and 29 kDa were identified after immuno-precipitation with Mab/a18. In agreement with other studies, the first could be attributed to the δ-opiate receptor [15,16] with the other two being ascribed to the μ-opiate receptor [17]. With respect to the latter, Bidlack and Denton [17] reported an apparent molecular mass of 35 kDa for the μ-opiate receptor, which was isolated by monoclonal antibody affinity chromatography [17]. In their study, however, a pre-purified mixture of proteins, and not a whole membrane extract, was used for affinity purification. Therefore, it is possible that the missing 29 kDa protein has been overlooked and, in this context, it should be noted that binding studies have indeed indicated the existence of multiple μ-opiate receptors [22]. The two species (36 and 29 kDa) identified in our study may therefore represent the μ_1- and μ_2-opiate receptors, an assignment which remains to be verified by further experiments.

The cross-reactivities of Mab/a9 and Mab/a18 between S_2-serotonin, D_2/D_4-dopamine, as well as δ- and μ-opiate receptors, reflect a certain structural homology in the ligand-binding site of these receptors. This homology might be implicated in basic processes of neurotransmission.

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