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Antibodies to the Cloned μ-Opioid Receptor Detect Various Molecular Weight Forms in Areas of Mouse Brain

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SUMMARY

Polyclonal antibodies directed against the amino-terminal portion of the cloned rat μ-opioid receptor (μOR) were raised in rabbits. The antibodies diminished the specific binding of ^{125}I-Tyr^{27}-β-endorphin-(1–31) (human) and [^{3}H][D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin, but not that of the δOR-selective ligand [^{3}H][D-Pen^{2,5}]enkephalin, to mouse brain membranes. The intracerebroventricular administration to mice of affinity-purified anti-μOR IgGs impaired the antinociception produced by the μOR agonists [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin and morphine and the μ/δOR agonists β-endorphin-(1–31) and [D-Ala^2,D-Leu^5]enkephalin, when studied 24 hr later in the tail-immersion test. Antinociception produced by the δOR-selective agonists [D-Pen^{2,5}]enkephalin and [D-Ala^2]deltorphin II was fully displayed in these mice. Immunoblots of sodium dodecyl sulfate-solubilized membranes from mouse central nervous system regions revealed protein bands of M_r 43,000, 51,000, and 58,000. Also detected were bands of higher molecular weights, 100,000 and 114,000, which probably corresponded to dimeric forms, because they disappeared after sonication of the solubilized tissues. This immunoreactivity was present in regions of mouse central nervous system and was barely detected in NG108–15 cells. After treatment of the solubilized material with endoglycosidase F, the antibodies labeled a band of M_r 43,000, coincident with the weight of the cloned μOR. These results confirm the existence of several molecular forms of the μOR due to glycosylation.

Determination of the primary structure and functional expression of a μOR from rat brain have recently been reported (1–4). The corresponding cDNA encodes a protein of 398 amino acids that binds pharmacologically defined μ ligands with greater affinity than those of the δ or κ types (1–4). In COS-7 cells expressing this receptor, DAMGO decreased the forskolin-stimulated production of cAMP, thus suggesting an inhibitory coupling to adenylyl cyclase via transducer G proteins (1). A molecular mass of 43/44 kDa was deduced from the reported sequence. However, greater molecular weight forms have consistently been reported for this OR, i.e., M_r 58,000 (5–7), 61,000 (8), 65,000 (9–11), and 35,000–108,000 (12–16). The *N*-linked glycosylation of this receptor seems to account for most of these different molecular weight forms (6, 15, 16). Putative *N*-linked glycosylation sites have already been described for the cDNA-deduced amino acid sequence of this μOR (1–4).

We have raised specific antibodies against the amino-terminal sequence of the cloned μOR. The affinity-purified IgGs reacted with proteins of 43–58 kDa in rat and mouse brain membranes and reduced the specific binding of [^{3}H]DAMGO and ^{125}I-Tyr^{27}-β-endorphin (human), but not [^{3}H]DPDPE, to mouse brain P_2 fractions. The intracerebroventricular injection of these IgGs into mice diminished the supraspinal antinociception produced by μ-selective agonists but not that produced by δ-selective ligands. The distribution of this μ-like immunoreactivity was determined in different areas of mouse brain, as well as in peripheral organs used for opioid pharmacological studies.

Experimental Procedures

Materials

The following substances were used. (3-[^{125}I]Iodotyrosyl^{27})-β-endorphin (human) (IM.162, 2000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). [Tyrosyl-2,6-^{3}H(N)]DPDPE (NET-922) (33.0 Ci/mmol) and [tyrosyl-3,5-^{3}H(N)]DAMGO (NET-902) (52.5 Ci/mmol) were obtained from DuPont de Nemours (Dreiech, Germany). Human β-endorphin-(1–31), DAMGO, DPDPE, DADLE, and [D-Ala^2]deltorphin II were purchased from Peninsula Laboratories (San Carlos, CA). SDS, mercaptoethanol, and chemicals for electrophoresis and immunoblotting were purchased from Bio-Rad (Madrid, Spain). Leupeptin, aprotinin, bacitracin, phenylmethylsulfonyl fluoride, EDTA, BSA, and prestained and biotinylated

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ABBREVIATIONS: OR, opioid receptor; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TTBS, Tris-buffered saline with 0.05% Tween-20; BSA, bovine serum albumin; PAG, periaqueductal grey matter.

protein standards were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Antibody production and purification. The peptide used was MDSSTGPGNTSDCSDP (BioSynthesis), corresponding to the 16 amino-terminal residues of the cDNA-predicted sequence (1–4). This particular amino acid sequence exhibited <30% homology to sequences in any known protein (in the EMBL, GenBank, and SwissPROT databases). Antisera MAS/1 and MAS/2 were raised in New Zealand White rabbits (Biocentre, Barcelona, Spain) using a conjugate [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] (Sigma E-6383) of the synthetic peptide and bovine thyroglobulin (Sigma T-1001) as antigen. The procedure described by Harlow and Lane (17) was followed. Blocking of free amino groups was achieved by incubating the carrier (2 ml of a 10 mg/ml solution) with 2 ml of 10 mg/ml citraconic anhydride, pH 8–9, for 1 hr. Blocked thyroglobulin was separated from free anhydride on a G-25 gel filtration column equilibrated with 100 mM phosphate-buffered saline, pH 8.5. The 20 mg of blocked thyroglobulin plus 80 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were incubated in 10 ml, pH 8.0, at room temperature for 30 min. The synthetic peptide (4 mg) was then added, with gentle stirring, to the activated carrier and incubated overnight at 4°. The conjugates were separated from free peptides by means of a G-25 gel filtration column and were concentrated using cellulose ester membranes (15,000 molecular weight cut-off) and the Micro-ProDiCon system (Spectrum).

Each rabbit received intradermal injections into 10–12 sites along its upper sides, plus a subscapular injection (0.3 ml), of an emulsion of 1 ml of the coupled peptide (1 mg) mixed with an equal volume of complete Freund's adjuvant (Calbiochem 344289). After 4–5 weeks animals were bled and boosted again the next week (18). Immunoreactivity was observed from the first bleeding. Preimmune IgGs were purified by using Protein A chromatography cartridges (MemSep CAPA 100 01; Millipore). The Protein A cartridge was equilibrated with 50 ml of 25 mM Tris·HCl, 1 M NaCl, pH 7.7. Four milliliters of crude serum were diluted (1/5) with this buffer, filtered through Millex GS filters (0.22 μm), and loaded onto the cartridge. Sample recirculation at 1 ml/min was continued for 60 min. The device was rinsed with equilibration buffer (2 ml/min) until the absorbance (280 nm) of the effluent reached the base-line. The bound IgGs were detached by passing 0.2 M glycine-HCl, pH 2.5, through the cartridge. The eluate was collected in a siliconized tube (Sigmacote) containing 2 ml of 1 M Tris·HCl, pH 7.7, until the monitored absorbance was at base-line (typically 10 ml). The eluted IgGs were dialyzed/concentrated to 500 μl against 2 × 5 liters of 10 mM Tris·HCl, 100 mM NaCl, pH 7.5, in a Micro-ProDiCon system (15,000 molecular weight cutoff; Spectrum); the final protein concentration of these preparations was about 0.5 μg/μl. To purify anti-μOR IgGs, the antigenic peptide was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Briefly, 2 g of affinity beads were swelled and washed in 400 ml of 1 mM HCl. Two milligrams of the antigenic peptide were dissolved in 10 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and mixed with the gel, by end-over-end rotation, for 24 hr at 4°. The gel was packed in an 8-ml column and the unbound peptide was washed away with 30 ml of coupling buffer. The remaining active groups were blocked by recirculation of 0.1 M Tris·HCl, pH 8.0, for 2 hr. Three wash cycles using 30 ml of 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0, followed by 30 ml of 0.1 M Tris·HCl, 0.5 M NaCl, pH 8.0, were performed. The anti-μOR IgGs were purified following the procedure described for Protein A cartridges. The dialyzed/concentrated IgGs gave a protein concentration of about 1.7 μg/μl.

Preparation of membranes from mouse brain and vas deferens, rat vas deferens, guinea pig vas deferens and ileal myenteric plexus, and NG108–15 cells. Albino, male, CD/1 mice (Charles River, Barcelona, Spain) weighing 22–27 g were used. Homogenates were prepared as described previously (19). Briefly, mice were killed by cervical dislocation. The brains were dissected into several areas and spinal cords were washed in ice-cold 50 mM Tris·HCl, 0.32 M sucrose, pH 7.5, at 4°. The pooled structures from 10 mice were homogenized with a Polytron homogenizer (model PT 10–35), at a setting of 3, for 15 sec and centrifuged at 1000 × *g* at 4° for 10 min. After the pellet was discarded, the supernatant was centrifuged at 20,000 × *g* for 20 min. That pellet was resuspended in buffer and centrifuged at 20,000 × *g* for an additional 20 min. The final pellet (P_2) was diluted in Tris buffer to a final protein concentration of 2 μg/μl. NG108–15 cells, vas deferentia from CD/1 mice, Wistar rats, and Hartley guinea pigs, and the myenteric plexus-longitudinal muscle of guinea pig ilea were processed as described above.

Treatment of mouse brain membranes with endoglycosidase F. The method described by Eppler *et al.* (16, 20) to deglycosylate the μOR was followed. Whole-brain P_2 fractions were centrifuged at 20,000 × *g* for 10 min. The membrane pellet was resuspended in 100 mM NaH_2PO_4, pH 6, 1 mM EDTA, 1% β-mercaptoethanol, 0.1% SDS, to 1 μg/μl final protein concentration and was heated at 100° for 3 min. The solubilized material was supplemented with 2% Triton X-100 and incubated with endoglycosidase F (EC 3.2.1.96; 0.25 units/50 μg of protein) for 18 hr at 37°. The samples were then concentrated by $CHCl_3$/methanol extraction, solubilized in sample buffer (2 μg of SDS/μg of protein) with 5% β-mercaptoethanol, and separated on a 7–18% SDS-polyacrylamide gel.

Electrophoresis and immunoblotting. Membrane samples were solubilized in a buffer containing 50 mM Tris·HCl, 3% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 6.8, and resolved by SDS-PAGE (gradient of 7–18% total acrylamide concentration/2.9% bisacrylamide cross-linker concentration) with 8-cm × 11-cm × 0.15-cm slab gels (Hoefer SE 280) at 15-mA constant current (ISCO power supply, model 595). Proteins were transferred (Mini-Trans-Blot electrophoretic transfer cell; Bio-Rad) to 0.2-μm polyvinylidene difluoride Trans-Blot membranes (Bio-Rad) using Towing buffer (25 mM Tris·HCl, 192 mM glycine, 0.04% SDS, 20% methanol), by application of 70 V (200–300 mA) for 120 min. Unoccupied protein binding sites were blocked with nonfat dry milk (M7439C; Bio-Rad) in TBS for 1 hr at 37°. Affinity-purified anti-μOR IgGs (2 μg/ml in TTBS) were added and incubated at 4° for 24 hr (unless otherwise stated) (Deca-Probe Incubation Manifold PR 150; Hoefer). After removal of the antibody, the blot was washed with TTBS. Secondary antiserum [goat anti-rabbit IgG (Fc)-alkaline phosphatase conjugate (S373B; Promega)] in TTBS was added at 1/3000 dilution and left for 3 hr. The secondary antiserum was removed and the membrane was washed as before with TTBS. Western Blue (S384B; Promega) (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide) was used as substrate.

Binding studies. Homogenates of mouse brain were prepared as described and supplemented with a mixture of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 0.5 μg/ml aprotinin). Membranes were incubated in the absence or presence of crude preimmune or immune serum (at 1/20 dilution, 1 μl of serum/30 μg of membrane protein) at 37° for 2 hr. After centrifugation at 20,000 × *g* for 20 min, the pellets were resuspended for binding assays. For ^{125}I-Tyr^{27}-β-endorphin (human), 4-ml siliconized (Sigmacote) borosilicate glass tubes were used (adsorption of the iodinated peptide was reduced to <5%). The incubation mixture consisted of 100 pM ^{125}I-Tyr^{27}-β-endorphin (competition assays with varying ICI-174,864 concentrations) or concentrations of ^{125}I-Tyr^{27}-β-endorphin ranging from 1 pM to 3 nM in the presence of 300 nM ICI-174,864 (saturation assays), membrane suspension at a final concentration of 0.4 mg/ml, 0.2% BSA, and 0.01% bacitracin, and the volume was made up to 2 ml with 50 mM (final concentration) Tris·HCl buffer, pH 7.5. Nonspecific binding was assessed in the presence of 1 μM unlabeled β-endorphin. Samples were incubated in triplicate at 25° for 90 min, in a shaking incubator, and were filtered under vacuum through glass fiber disks (Whatman GF/B) that had been previously immersed for 3 hr in 5 mM Tris·HCl/0.3% polyethylenimine, to minimize binding to the filters. After filtration, the

filters were washed three times with 4 ml of ice-cold 5 mM Tris·HCl buffer, pH 7.5. Filters were placed in polyethylene counting vials and counted in a LKB Compugamma CS counter. For [^{3}H]DAMGO and [^{3}H]DPDPE, binding was performed as described for ^{125}I-Tyr^{27}-β-endorphin but in the absence of ICI-174,864, BSA, or bacitracin. For these ligands, concentrations ranging from 100 pM to 6 nM were studied. Nonspecific binding was assessed in the presence of 1 μM unlabeled naloxone. Radioactivity was counted in a Beckman LS-5801 scintillation counter.

Evaluation of antinociception. Albino, male, CD-1 mice (Charles River) weighing 22–25 g were used throughout. Animals were kept at 22°, with a 12-hr light/dark cycle (lights on from 8 a.m. to 8 p.m.), and maintained with food and water provided *ad libitum*. To reduce the possibility of reaching spinal levels, all substances were administered intracerebroventricularly to ether-anesthetized mice, in a final volume of 4 μl. Injections were unilateral. The tail-immersion (52°) test was used to evaluate analgesia. Latencies (in seconds) were measured manually, both before treatment (basal latency) and after the administration of the opioid agonist under study. A cut-off time of 10 sec was selected and the antinociceptive activity was expressed as a percentage of the maximum possible effect (MPE). The following equation was used: % MPE = 100 × (test latency − basal)/(10 − basal). A single dose of saline (control), Protein A-purified preimmune IgGs, or affinity-purified IgGs was injected into the mice and then the antinociceptive effect of the analgesic substances was evaluated.

Statistical significance was determined by analysis of variance, followed by the Newman-Keuls test. The level of significance was set at $p < 0.05$.

Protein determination. The protein content of the solubilized samples was determined by the method of Lowry *et al.* (21), after precipitation with 0.15% (w/v) deoxycholic acid and 72% (w/v) trichloroacetic acid (22).

Results

Reduction by anti-μOR of the specific binding of ^{125}I-Tyr^{27}-β-endorphin-(1–31) and [^{3}H]DAMGO. The δOR-selective antagonist ICI-174,864 exhibited successive inhibition of 0.1 nM ^{125}I-Tyr^{27}-β-endorphin-(1–31) binding to δORs and μORs in mouse brain membranes (Fig. 1). A concentration of 300 nM ICI-174,864, which abolished the binding of the iodinated neuropeptide to the δOR, was selected for the following assays. The affinity displayed by ^{125}I-Tyr^{27}-β-endorphin-(1–31) and DAMGO for membranes that had been preincubated with the antipeptide antibody MAS/1 was significamtly reduced (Fig. 1). This binding was fit to a single site (23). The K_d values and 95% confidence limits displayed by ^{125}I-Tyr^{27}-β-endorphin-(1–31) for this site in control (no serum), preimmune serum-, and anti-μOR-treated membranes were 0.30 (0.33–0.27) nM, 0.28 (0.31–0.24) nM, and 0.50 (0.60–0.40) nM, respectively. The μOR-selective ligand [^{3}H]DAMGO displayed K_d values of 0.40 (0.44–0.35) nM and 0.69 (0.76–0.62) nM for this receptor in membranes that had been incubated with preimmune serum (or no serum) and anti-μOR serum, respectively (Fig. 1). The maximum binding

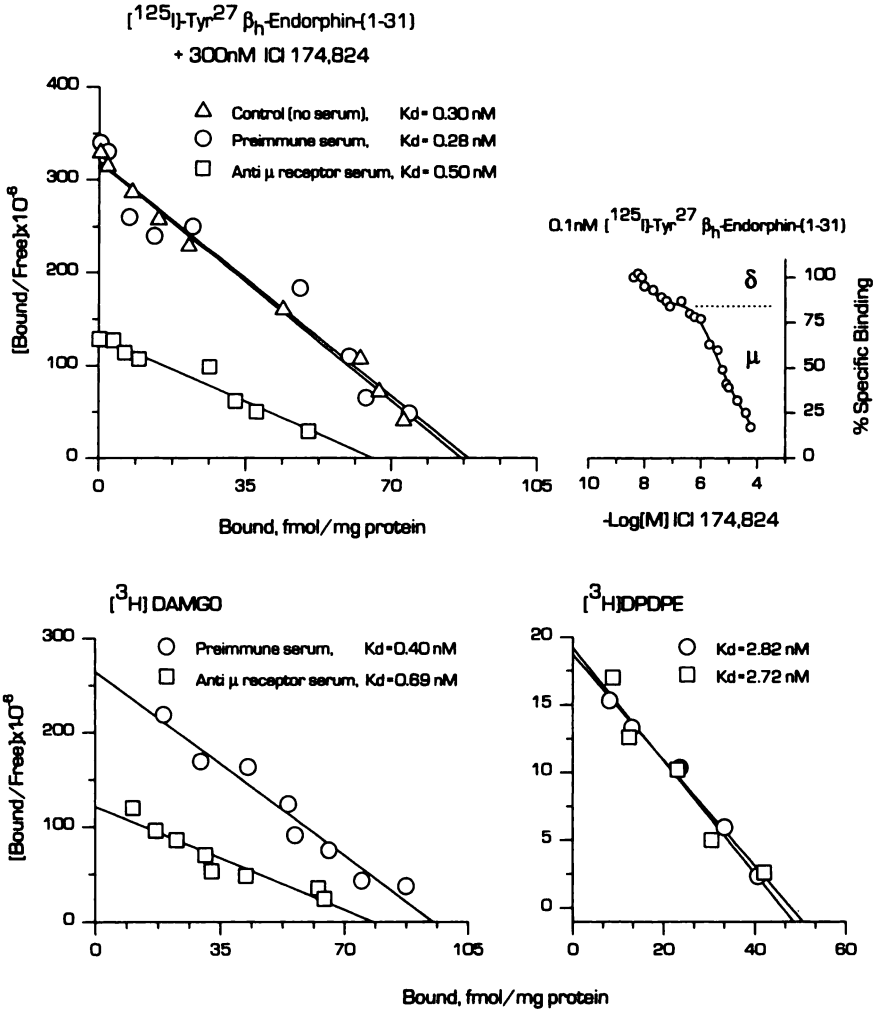

Fig. 1. Specific binding of opioid agonists to mouse brain membranes (minus cerebellum and medulla). Scatchard plots of [^{3}H]DAMGO, [^{3}H]DPDPE, and ^{125}I-Tyr^{27}-β-endorphin-(1–31) binding to membranes that had been preincubated at 37° for 2 hr, in the absence or presence of preimmune serum or anti-μOR serum (MAS/1), are shown. Increasing concentrations of the opioid agonists were incubated with approximately 0.25 mg/ml final protein concentration at 25° for 90 min. In the competition experiment, 0.1 nM ^{125}I-Tyr^{27}-β-endorphin-(1–31) was incubated with various concentrations of unlabeled ICI-174,824. Binding parameters (see text) were computed from three independent experiments.

capacity of both ligands for membranes incubated with the anti-μOR serum appeared significantly diminished [from 87 (99–74) to 62 (70–54) fmol of ^{125}I-Tyr^{27}-β-endorphin-(1–31)/mg of protein and from 96 (108–83) to 71 (81–61) fmol of [^{3}H]DAMGO/mg of protein; average and 95% confidence limits]. The binding of [^{3}H]DPDPE (a δOR-selective ligand) to membranes exposed to anti-μOR serum remained unchanged (Fig. 1).

Effect of *in vivo* intracerebroventricular administration of anti-μOR (MAS/2) on the supraspinal analgesia induced by various opioid agonists in the tail-immersion test. Two micrograms of the affinity-purified immune IgGs (1.7 μg/μl) were intracerebroventricularly injected into the mice, in 4 μl. Control animals received saline or 2 μg of Protein A-purified IgGs (preimmune serum) in 4 μl. The supraspinal antinociceptive activity of a series of opioid agonists was evaluated 24 hr later. At this interval, the mice receiving the anti-μOR showed a slight but significant increase of basal latencies when evaluated in this analgesic test (1.66 ± 0.06 and 2.35 ± 0.06 sec in control and antiserum-treated mice, respectively; mean ± standard error; n = 25). No motor disturbances were observed. Opioid agonist-induced supraspinal antinociception was fully expressed in those animals given intracerebroventricular injections of either saline or preimmune IgGs. The potency of the preferential μOR ligands DAMGO and morphine and of the μ/δOR agonists β-endorphin-(1–31) and DADLE appeared reduced, although to a limited extent, in mice receiving MAS/2 IgGs (Fig. 2). Antagonism produced by a single intracerebroventricular injection of the anti-μOR lasted for at least 48–72 hr. Similar results were observed for antibody dilutions of 0.2 μg/4 μl; higher dilutions lacked this inhibitory effect on μ-mediated analgesia (data not shown). The analgesic effect of the δ-selective ligands DPDPE and [D-Ala^2]deltorphin II showed no changes (Fig. 2).

Immunoblots of mouse central nervous system regions and NG108–15 cells with anti-μOR IgGs. Immunoblots of SDS-solubilized rat and mouse PAG membranes (30 μg of protein/lane) showed immunoreactive bands at molecular masses of 43, 51, 58, 100, and 114 kDa (Fig. 3). The upper bands disappeared when samples were sonicated for 10 min during the solubilization procedure (Fig. 3). Therefore, dimerization of lower molecular mass forms appears to take place. These aggregates have been previously described during purification of rat brain μORs (6). The immunosignals were absent when Protein A-purified IgGs of preimmune serum were used (2 μg/ml TTBS). Moreover, preincubation of affinity-purified anti-μOR IgGs (2 μg/ml TTBS) with the immunogenic peptide (0.5 mg for 1 hr at room temperature) reduced the immunosignals (Fig. 3). After SDS-PAGE (70 μg of protein/lane) and immunoblotting, bands of molecular weight 43,000, 51,000, and 58,000 were immunodetected in extracts of tissues from various areas of mouse brain, with the M_r 51,000 form being predominant (Fig. 4). The rat and guinea pig vas deferentia presented higher levels of immunoreactivity than did the mouse vas deferens. Also, high levels of μ-like immunoreactivity were detected in the myenteric plexus-longitudinal muscle preparation of the guinea pig ileum (Fig. 4).

The cell line NG108–15, derived from a murine neuroblastoma, is reported to contain mainly δORs. SDS extracts of this cell line showed no μ-like immunoreactivity (or faint bands at M_r 43,000/58,000) (Fig. 5). The Δ/1 antiserum directed against the first 16 amino acids of δOR amino-terminal sequence (24) detected two bands, of 39 and 45 kDa. The lower band coincided with the δOR immunodetected in regions of mouse brain (24). The upper band was not found in mouse brain and might be due to glycosylation of the M_r 43,000 δOR.

The molecular weights of the μOR-related M_r 51,000 band and the upper bands, including the M_r 105,000 band, decreased to M_r 43,000 after treatment of solubilized whole-brain membranes with endoglycosidase F (Fig. 6). No sonication was applied to these samples. As suggested previously (6, 15, 16), glycosylation seems to account for the molecular forms of the μOR larger than 43 kDa.

Discussion

The polyclonal antisera raised against the first 16 amino acids of the amino-terminal sequence of cloned rat μOR proved useful for recognizing the corresponding receptor in mice. As observed for the δOR (2, 25, 26), the μORs of these two species should share most of the amino-terminal region. However, in other species (rats and humans) certain differences have been described (27). These antipeptide antibodies

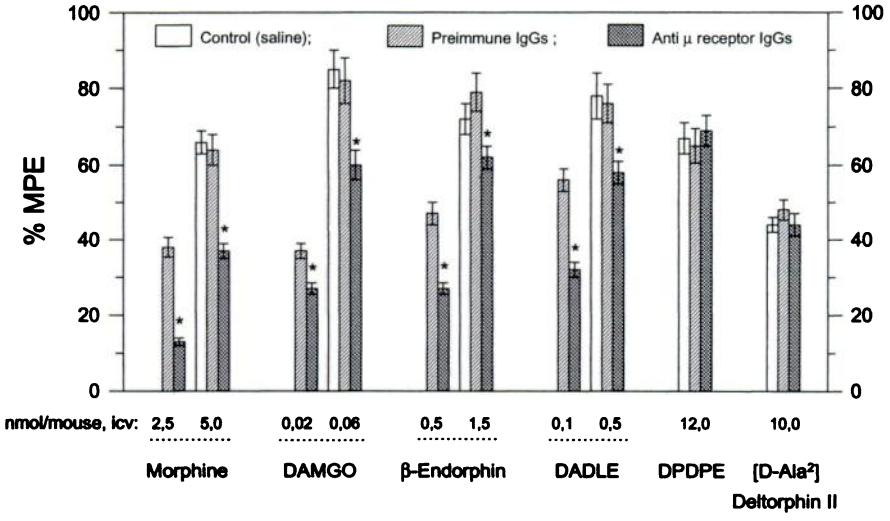

Fig. 2. Effect of intracerebroventricular administration of anti-μOR (MAS/2) antibody to mice on opioid agonist-induced supraspinal antinociception in the tail-immersion test. Mice received a single injection of saline (control) or 2 μg of affinity-purified IgGs or Protein A-purified preimmune IgGs in 4 μl. Opioid agonists were injected intracerebroventricularly 24 hr later, at the doses indicated, and antinociception was determined at its peak, i.e., 30 min after morphine or β-endorphin, 15 min after DAMGO, DADLE, or DPDPE, or 10 min after [D-Ala^2]deltorphin II. Analgesia is expressed as a percentage of the maximum possible effect (% *MPE*) detected in this test, with a cut-off time of 10 sec. Values are the mean ± standard error from groups of 15–20 mice. *, Significantly different from the control group (analysis of variance followed by the Newman-Keuls test, $p < 0.05$).

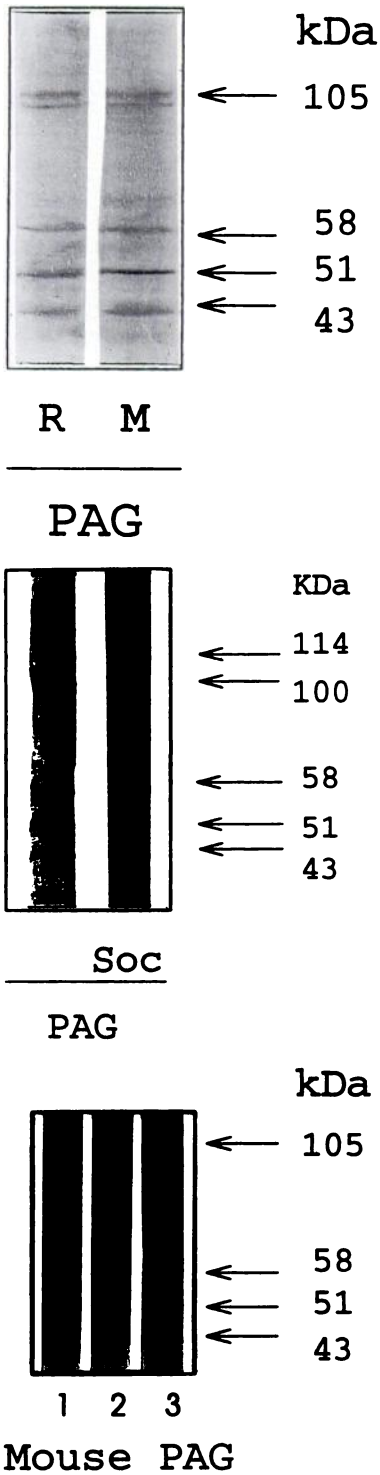

Fig. 3. Immunoblots of SDS extracts from mouse and rat PAG with anti-μOR antibody. SDS-solubilized samples (30–40 μg/lane) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with affinity-purified MAS/1 IgGs as described. The signal was produced by the action of alkaline phosphatase, conjugated to goat anti-rabbit IgG, on the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate. *Upper*, *R*, rat PAG; *M*, mouse PAG; *middle*, mouse PAG solubilized with (*Soc*) and without sonication for 10 min; *lower*, *lane 1*, preimmune IgGs; *lane 2*, MAS/1 IgGs incubated with the antigenic peptide; *lane 3*, MAS/1 IgGs. The molecular weight standards used were α^2-macroglobulin (M_r 190,000), β-galactosidase (M_r 125,000), fructose-6-phosphate kinase (M_r 88,000), pyruvate kinase (M_r 65,000), fumarase (M_r 56,000), lactic de

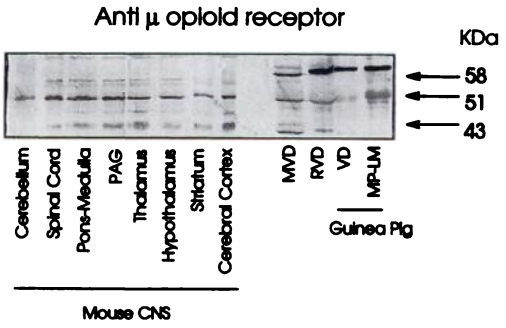

Fig. 4. Immunoblots of SDS extracts from various mouse brain regions and peripheral organs of various species, with anti-μOR antibody. Tissues were solubilized with SDS and 70 μg/lane were electrophoresed. Immunoblots with affinity-purified MAS/1 were carried out as described. MP-LM = myenteric plexus-longitudinal muscle, MVD = mouse vas deferens, RVD = rat vas deferens, VD = vas deferens.

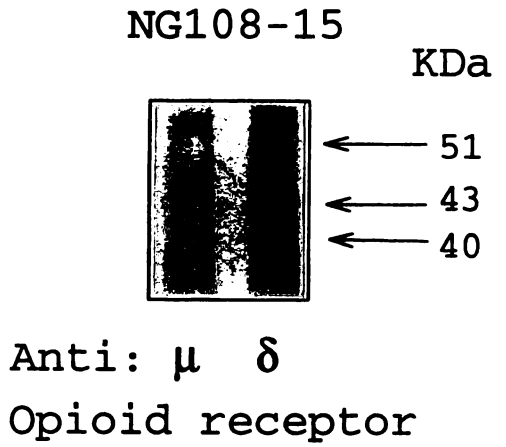

Fig. 5. Immunoblots of SDS extracts from NG108–15 cells, with anti-μOR and anti-δOR antibodies. SDS-solubilized samples (50 μg/lane) were resolved by SDS-PAGE and electroblotted, and immunoblots were performed as described. The MAS/1 and Δ/1 affinity-purified IgGs were used at 2 μg/ml of TTBS.

reduced the specific binding of labeled DAMGO and β-endorphin-(1–31) to mouse brain membranes, without altering the binding profile of the δ-selective ligand DPDPE. It was recently reported that μORs lacking most of the amino terminus still displayed strong binding of DAMGO (27). However, the attachment of the IgGs to this region of the μOR reduced the binding not only of the longer endogenous neuropeptide β-endorphin but also of the synthetic pentapeptide DAMGO. The purified IgGs also exhibited selectivity for the μ-mediated supraspinal analgesia elicited by opioid agonists in mice. The binding of these antibodies to the pharmacologically defined μOR is therefore suggested.

Glycosylation of the μOR has been shown (15, 16). The cloned μOR exhibited several sites for *N*-linked glycosylation, showing certain variations among species (15, 27). We found an immunoreactive band of M_r 43,000 that coincided with the deducted molecular weight of the cloned receptor. Other bands of higher molecular weight, 51,000 and 58,000,

hydrogenase (M_r 38,000), and triose phosphate isomerase (M_r 33,000). Biotinylated SDS molecular weight markers were phosphorylase *b* (M_r 97,000 subunit), catalase (M_r 58,100 subunit), alcohol dehydrogenase (M_r 39,000 subunit), carbonic anhydrase (M_r 29,000), trypsin inhibitor (M_r 20,100), and lysozyme (M_r 14,300).

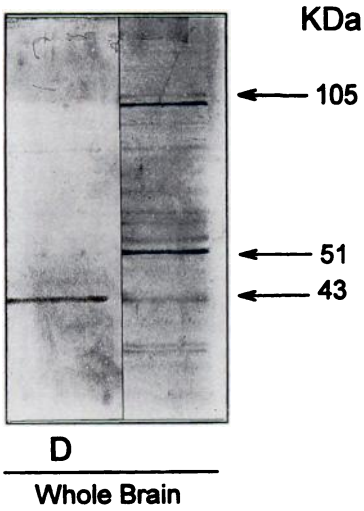

Fig. 6. Effect of deglycosylation on immunoblots from SDS extracts of mouse whole brain, with anti-μOR IgGs. Solubilized membranes were deglycosylated with endoglycosidase F as described, separated by SDS-PAGE (50 μg/lane) on 7–18% gels, and immunodetected with MAS/1 antibody. *Lane D*, deglycosylated material; *Right Lane*, control (nondeglycosylated) material.

might represent different degrees of glycosylation or an apparent heterogeneity of molecular weight, mostly due to differences in mobility of the glycoprotein in the polyacrylamide matrix. Carbohydrate side chains of glycoproteins may have charged residues, i.e., sialic acid or sulfate, affecting SDS binding and consequently their migration rate in SDS-PAGE. Glycoproteins display anomalous mobility due to reduced binding of detergent on a weight basis, compared with proteins of the same mass. This lower mobility is most pronounced at low acrylamide concentrations (for example, see Ref. 18). These considerations might account for the diverse masses described for this glycosylated receptor (see the introduction). As noted previously (6), glycosylated receptors might form dimers of M_r 100,000 or 114,000 (Refs. 13 and 14 and the present paper). Although the antigenic peptide includes a putative site for glycosylation, i.e., residue 9, the polyclonal antisera still recognized several glycosylated forms. However, other receptors not labeled by this antipeptide antibody might exist. Glycosylated receptors (68–97 kDa) belonging to several species, including mice, were labeled by β-[^{3}H]funaltrexamine; however, no labeling of the M_r 43,000 receptor was reported before the removal of the sugars with *N*-glycanase (15). It is possible that nonglycosylated μORs bound to β-funaltrexamine were missed during purification before SDS-PAGE analysis. There are reports in the literature describing both sensitive and insensitive (to the blocking effect of β-funaltrexamine) μORs (28, 29). Thus, β-funaltrexamine may bind irreversibly only to glycosylated ORs.

Naloxonazine binds with high affinity to the cloned μOR (1, 4), suggesting that this receptor corresponds to the pharmacological μ_1 subtype (30). Opioid agonists are reported to display high affinity at the μ_1 subtype (31), although other authors showed that the cloned receptor fits best with the μ_2 subtype (3). High affinity of agonists strongly depends on the coupling of the receptors to transducer G proteins. It is possible that those cell lines expressing the cloned receptors bear a low concentration of or even lack a critical G protein. The multiplicity of G proteins regulated by μORs has already been documented, i.e., G_i, G_o, and $G_{x/z}$ (7, 32, 33). It will be of interest to explore whether the μ_1 and μ_2 subtypes are related to regulation of different G proteins or reflect glycosylation variants of the cloned receptor or alternate splicing of the precursor mRNA (34).

The distribution of the immunoreactivity correlated well with the presence of μOR binding in areas of mouse central nervous system (35) and peripheral organs. Note the detection of weak immunoreactivity in the cerebellum; the μOR mRNA has been recently detected in this structure (36). The δOR also has been detected in discrete zones of the cerebellum. This is supported by the detection of autoradiographic grains (37), the gene (38), and immunoreactivity (24). The μ-like immunoreactivity was barely detected in the NG108–15 cell line. The rat vas deferens contains immunoreactive material, which might be interpreted as the ε- and μORs sharing the sequence of the antigenic peptide or μORs being present in this organ (39).

The present study showed μ-like immunoreactivity in regions of mouse central nervous system. Several bands of this glycosylated receptor were detected, which reasonably coincide with those molecular masses described previously. The possible pharmacological and physiological relevance of the distinct forms of the receptor merits consideration.

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