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Monoclonal antibodies against the native or denatured forms of muscarinic acetylcholine receptors

C.André*, J.G.Guillet[1], J.-P.De Backer, P.Vanderheyden, J.Hoebeke[1] and A.D.Strosberg[1]

Department of Protein Chemistry (subunit Pathological Biochemistry), Free University of Brussels, 65 Paardenstraat, 1640 Sint Genesius Rode, Belgium, and [1]Laboratory of Molecular Immunology, Jacques Monod Institute, C.N.R.S., University Paris VII, Tour 43, 2 Place Jussieu, 75251 Paris Cedex 5, France

*To whom reprint requests should be sent

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BALB/c mice were immunized with affinity-purified muscarinic acetylcholine receptors from calf brain and their splenocytes fused with NS1 myeloma cells. Hybrid cultures were grown and selected for production of antibodies on the basis of enzyme immunoassays on calf and rat forebrain membrane preparations. Thirty-four clones were retained and six of them further subcloned. Two of these subclones produced antibodies that selectively recognized muscarinic acetylcholine receptor-bearing membranes. The M-35b antibodies interacted only with native digitonin-solubilized receptors, and not with denatured receptors. The M-23c antibodies did not react with active digitonin-solubilized receptors but recognized the denatured form. The M-23c antibodies should thus be useful in the purification of the receptor and its precursor translation products, while the M-35b antibodies could be used for the immunocytochemical localization of the receptor in cells and tissues of different species.

Key words: hybridoma/Western blot/immunoprecipitation/monoclonal antibodies/muscarinic acetylcholine receptors

Introduction

The binding of the neurotransmitter acetylcholine elicits rapid responses via the nicotinic receptors and slower effects via the muscarinic receptors. Nicotinic receptors found in high concentrations in the post-synaptic membrane of the electromotor synapses of electric fish have been extensively characterized (Changeux, 1981). Partial sequencing of the polypeptide chains (Devillers-Thiery *et al.*, 1979; Raftery *et al.*, 1980) and cloning and sequencing of the corresponding genes (Noda *et al.*, 1982, 1983; Giraudat *et al.*, 1982; Claudio *et al.*, 1983) have allowed the elucidation of the complete structure of the receptor. The study of the mammalian nicotinic receptors is progressing rapidly along the same lines (Miledi *et al.*, 1982). Muscarinic acetylcholine receptors (mAChRs) were characterized in mammalian smooth muscle and brain tissues by the use of the radiolabeled antagonists dexetimide, quinuclidinyl benzylate and atropine (Yamamura and Snyder, 1974; Laduron *et al.*, 1979). Irreversible labeling was achieved using propylbenzilylcholine mustard (Birdsall *et al.*, 1979).

mAChRs were solubilized with a variety of detergents of which digitonin appeared to be the most effective (Hurko, 1978; Aaronstam *et al.*, 1978; Ruess and Lieflander, 1979; Repke and Mathies, 1980; Gorissen *et al.*, 1981). Affinity purification of a single protein of 70 kd from solubilized calf forebrain membranes was achieved using a gel containing dexetimide coupled to agarose. Elution from the gel was carried out using either atropine, or the irreversible ligand propylbenzilylcholine mustard (André *et al.*, 1983).

We report here the production of monoclonal antibodies raised against the affinity-purified receptor protein. These antibodies recognize mAChRs on cell membranes from various origins and immunoprecipitate either the active digitonin-solubilized mAChRs or the denatured mAChRs and the radioiodinated purified 70-kd protein.

Results

From the fusion of NS1 myeloma cells with the splenocytes of BALB/c mice immunized with the affinity-purified calf brain 70-kd mAChR, 34 clones were obtained whose secretion products reacted strongly with mAChR-bearing membranes but did not react with membrane preparations from P815 mastocytoma cells (Dunn and Potter, 1957), which are devoid of mAChR binding activity. Six of these were subcloned and two of the subclones, M-23c and M-35b, were further selected for production of ascites in mice (Table I). The ascites produced by both hybridomas were titrated against calf brain membranes. The M-35b ascitic fluids were 30 times more effective than those of M-23c in recognizing the membranes as shown in Figure 1.

To determine the specificity of the antibodies secreted by these two hybridomas, a Western blot was performed on a SDS-mercaptoethanol solubilized calf brain membrane preparation after polyacrylamide gel electrophoresis (PAGE). As shown in Figure 2, the M-23c IgC antibody revealed a band at 70 kd while M-35b, an IgM, did not show any specific staining. These results were confirmed using radioiodinated affinity-purified mAChRs (André *et al.*, 1983). While the M-23c antibody immunoprecipitated the 70-kd protein in high amounts, the M-35b antibody did not react with this protein in amounts higher than the background level precipitated by a non-specific monoclonal antibody

Table I. E.I.A. screening of monoclonal antibodies on mAChR-bearing membranes of various origins

Clones	Membranes			Solubilized Calf
	Rat	Calf	P815	
M-23c	0.20	1.23	0.14	0.50
M-35b	0.44	0.86	0.07	0.86
ns	0.04	0.20	0.15	0.12

Culture supernatants were screened in PVC Linbro microtiter plates coated with intact or solubilized membrane preparations. The antibody reactivity was indicated by immunoperoxidase staining (peroxidase-labeled rabbit anti-mouse IgG antibodies, ABTS-H_2O_2). Data given are total (incubation with culture supernatants) minus background (incubation with PBS) absorbances at 405 nm. 'ns' stands for 'non-specific' antibody and corresponds to an antibody unrelated to the present study.

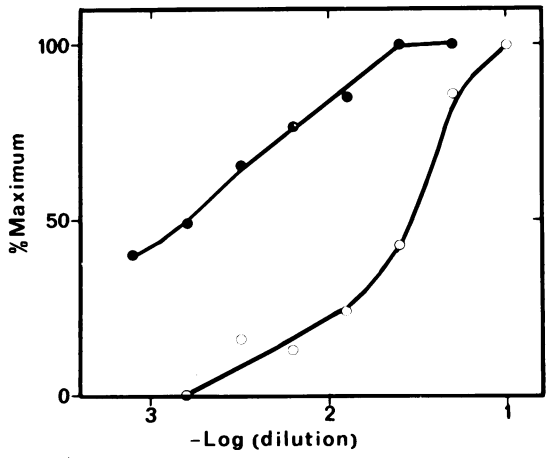

Fig. 1. Titration curves of M-23c and M-35b on calf brain membranes by E.I.A. The results are expressed as % of the maximal optical densities at 405 nm. All values were corrected for background, determined in the absence of monoclonals. M-23c (○——○) was revealed by a rabbit anti-mouse IgG and M-35b (●——●) by a goat anti-mouse μ chain conjugated to horseradish peroxidase.

(Figure 3).

The interaction of both antibodies with the native mAChR was tested in two ways. An indirect immunoprecipitation was performed using the digitonin-solubilized calf brain membranes as antigen source. The M-35b monoclonal antibody showed a 75% precipitation of dexetimide (antagonist) binding proteins, while the M-23c immunoprecipitated only 10% of the binding activity (Figure 4A). These results were confirmed by using as immunosorbent the ascitic proteins coupled to a CNBr-activated Sepharose 4B (Figure 4B). While the M-35b immunosorbent adsorbed 50% of the dexetimide binding activity from digitonin-solubilized calf brain membrane preparations, the M-23c immunosorbent did not retain any binding activity above background level.

The direct interaction of the two monoclonal antibodies with the binding site of the mAChR was tested by studying the inhibition of dexetimide binding to digitonin-solubilized mAChRs in competition binding experiments. Neither the M-23c nor the M-35b antibody significantly inhibited the antagonist binding to the receptor.

Discussion

In recent years pathologists have found that anti-receptor antibodies play a central role in the etiology of autoimmune disorders (Köhler, 1980; Strosberg, 1983). Moreover, anti-receptor antibodies have been found to be an excellent tool for the localization and the purification of receptors. The introduction of monoclonal antibody technology has increased the interest in the production of anti-receptor antibodies since monoclonal antibodies, each of which recognizes only a single antigenic determinant, can provide a panel of homogeneous reagents with different immunological and physiological properties. In the present study we have used mAChRs of calf brain, recently purified by affinity chromatography (André *et al.*, 1983), as an antigen to obtain monoclonal anti-receptor antibodies.

Of the 34 hybridoma supernatants which showed strong reactivity towards calf brain membrane preparations, six were subcloned and two of the subclones were grown in ascites. M35-b was most active on intact rat and calf brain membranes or solubilized calf brain membrane preparations as shown in Table I and Figure 1. Antibody M-23c reacted with solubilized as well as intact calf brain membranes but recognized only poorly the intact rat brain membranes. On the basis of these differences we initiated the study of these two monoclonal antibodies.

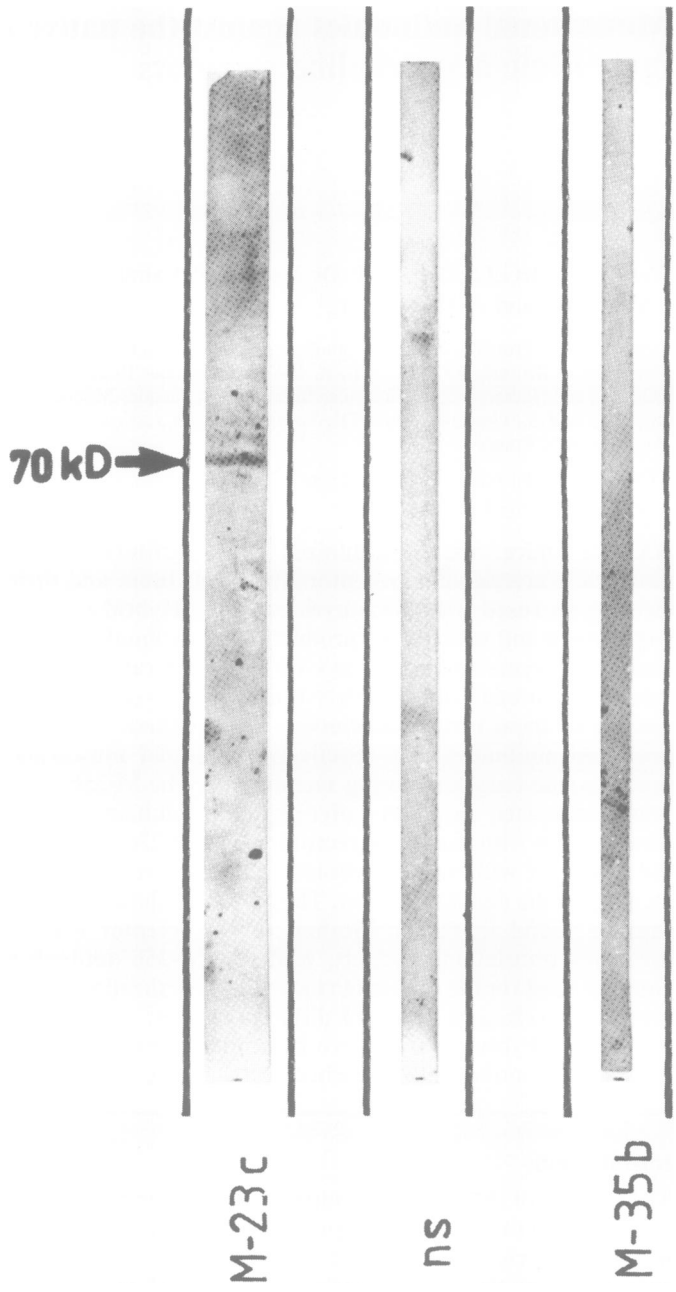

Fig. 2. Reactivity of monoclonal antibodies with SDS-PAGE analysed calf brain membrane proteins (Western blot analyses). After SDS-PAGE, subsequent blotting and final incubation with a 1:10 dilution of the ascites fluid, bound monoclonal antibodies were revealed by immunoperoxidase staining (peroxidase-labeled rabbit anti-mouse IgG antibodies, 4-chloro-1-naphthol/H_2O_2). Bound monoclonal antibodies appeared as dark blue bands. The mol. wt. of the immunoprecipitated band was evaluated by means of reference standards simultaneously analysed by SDS-PAGE, blotted and developed with Coomassie blue.

The monoclonal antibody M-23c was specific for the 70-kd protein, which carries the binding site of the mAChR of calf brain (André *et al.*, 1983). By immunoblotting, the antibodies recognized only a 70-kd protein among the membrane pro-

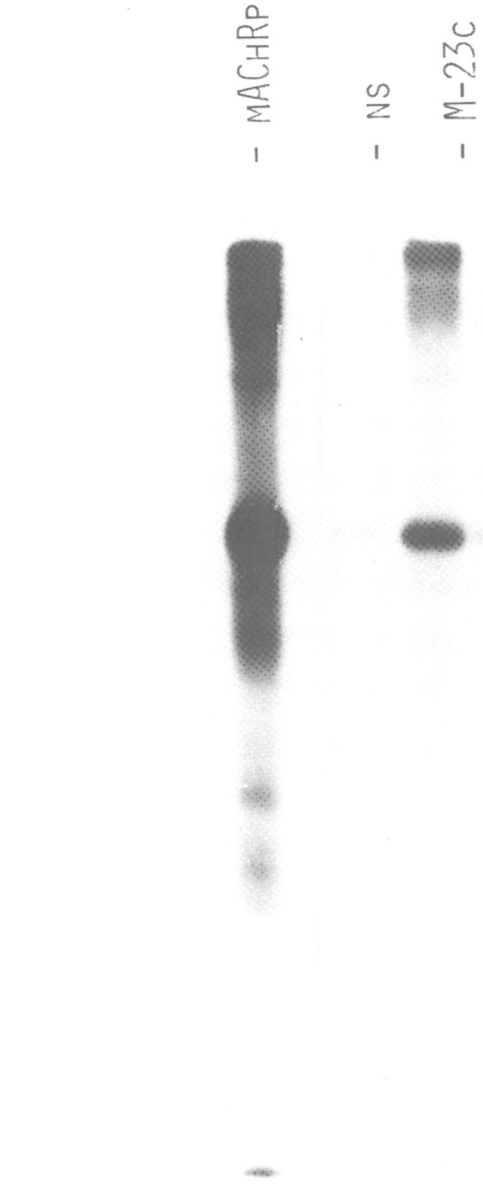

Fig. 3. Reactivity of monoclonal antibodies with the purified radioiodinated mAChR from calf brain. SDS-PAGE analysis of the immunocomplexes formed between the monoclonal antibodies or the 'non-specific' antibodies bound to protein A-Sepharose CL-4B gel particles, and the radioiodinated purified mAChR (mAChRp).

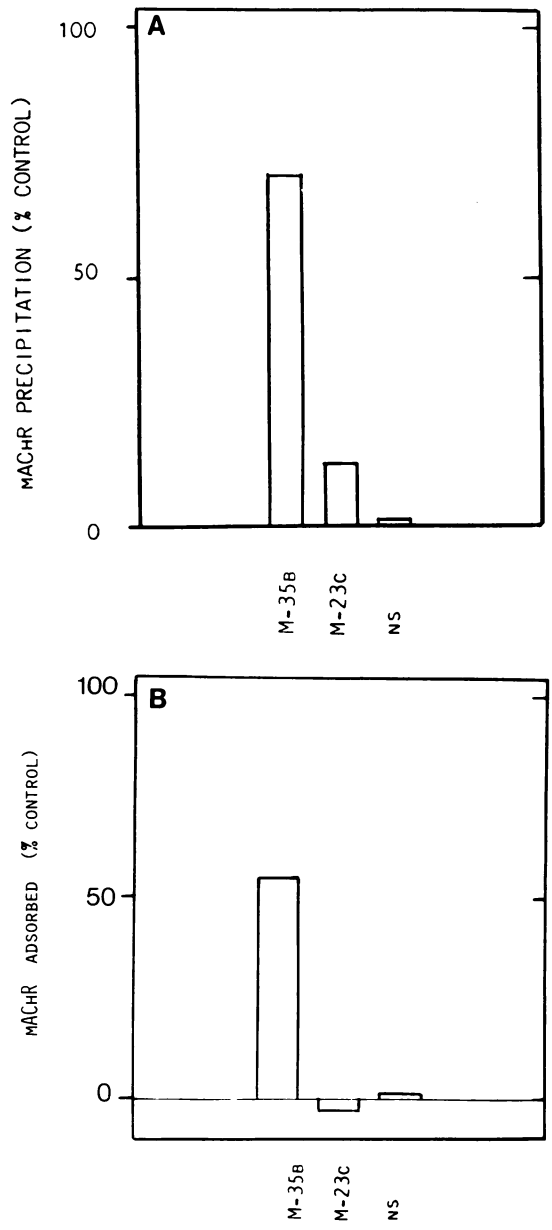

Fig. 4. Reactivity of the monoclonal antibodies with the native, digitonin-solubilized, mAChRs from calf brain. Percentages of specific [^{3}H]-dexetimide binding activity in the incubation medium after precipitation of the formed immunocomplexes. **A**: indirect immunoprecipitation via rabbit anti-mouse IgG antibodies and non-specific purified mouse IgG for network formation. **B**: direct immunoadsorption with monoclonal antibodies coupled to CNBr-activated Sepharose 4B.

teins; the identification of this protein as the muscarinic receptor was confirmed by the immunoprecipitation of the radioiodinated receptor, purified by affinity chromatography (André *et al.*, 1983). M-23c, which recognized solubilized calf brain membranes three times less than intact membranes, only weakly immunoprecipitated the native receptor, suggesting that the recognized antigenic determinant was reduced in concentration or in affinity during the solubilization procedure. The poor recognition of rat brain membranes suggests that the antigenic determinant recognized by M-23c is probably not responsible for the active conformation of the receptor, since one would expect that the active site is conserved in different species. The existence of minor structural dissimilarities in the mAChRs from the two animal species may however be invoked.

The monoclonal antibody M-35b was ineffective in recognizing the immunoblotted or the radioiodinated receptors. It was most effective in recognizing the receptors from the solubilized membrane preparation, as shown by direct immunoadsorption or indirect immunoprecipitation. Both of these facts suggest that the antigenic determinant recognized by M-35b is a conformational determinant present only on the active receptor. Two findings indicate that this conformational determinant is important for the function of the mAChRs: (i) the determinant appears to be conserved on mAChR of other species since rat membranes are very well recognized by the M35-b antibody, and (ii) preliminary results

indicate that this antibody has a marked carbachol-like (agonist-like) behaviour on the cyclic GMP content of guinea-pig myometrium (Leiber and Harbon, 1982; Leiber *et al.*, in preparation). This effect is probably not due to a direct binding of the antibody to the binding site of the hormone since no competition between M-35b and dexetimide for the same binding site could be demonstrated.

To summarize, using BALB/c mice immunized with affinity-purified mAChRs, we have prepared two monoclonal antibodies which recognize the mAChR of calf brain. One of these, specific for an antigenic determinant on the 70-kd protein in its inactive form, should be useful for the immunopurification of the receptor and its precursor translation products. The second, which recognizes a conformational determinant on the active receptor, will be used for immunocytochemical localization of the receptor in different tissues of various species. Its ability to trigger physiological effects should lead to new approaches in the study of the mAChR functions.

Materials and methods

Chemicals

Atropine sulphate monohydrate was obtained from Aldrich Europe, [^{3}H]-dexetimide (14 Ci/mmol) from I.R.E. (Fleurus, Belgium) and [^{3}H]propylbenzilylcholine mustard (44 Ci/mmol) from New England Nuclear (Dreieich, FRG). Polyethyleneglycol (PEG 2000) was purchased from Merck (Darmstadt, FRG) and poly-L-lysine (mol. wt. 70–150 kd), bovine serum albumin (BSA) and 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) from Sigma Chemical Company (St. Louis, USA).

Preparation of membrane-bound and digitonin-solubilized mAChRs

Intact rat or calf forebrain plasma cell membranes (~2.8 pmol mAChR/mg of protein) were prepared as previously described (André *et al.*, 1983; Vauquelin *et al.*, 1982). Solubilized mAChR preparations (~1 pmol mAChR/mg of protein) were made by continuously vortexing intact membranes with digitonin (final 10 mg/ml suspension) for 25 min at 4°C followed by centrifugation in a Sorvall centrifuge (SS34 rotor) at 15 000 r.p.m. for 30 min at 4°C. P815 mastocytoma cells were lysed by rapid decompression after equilibration for 10 min with N_2 at 50 bar in a Mini-Bomb Kontes cell disruption chamber. The membranes were precipitated at 105 000 *g*.

Affinity chromatography

mAChRs from calf forebrain plasma cell membranes were solubilized with a 1% digitonin (w/v) suspension, specifically absorbed on a dexetimide affinity gel and eluted with the selective muscarinic antagonist atropine or the irreversible ligand propylbenzilylcholine mustard as previously described (André *et al.*, 1983). Iodination of the affinity-purified receptor was carried out by the chloramine T method (Greenwood *et al.*, 1973).

Immunizations and cell fusions

BALB/c mice were immunized with affinity-purified mAChRs by a footpad injection of 0.1 pmol (~10 ng protein) in complete Freund's adjuvant, followed 3 weeks later by an i.v. booster injection of 0.1 pmol of purified mAChRs in phosphate buffered saline (PBS). Three days after the booster, spleen cells were fused with the non-secreting myeloma cell line NS1 according to a previously described procedure (Köhler and Milstein, 1975) in the presence of PEG 41%. The fused cells were distributed into four Linbro plates (24 two ml wells/plate); colonies developed in 84 wells.

Screening of the cell hybrids

The supernatants from the 84 hybrid cultures were tested for the presence of antibodies reacting selectively with mAChR-bearing membrane preparations in an initial screening procedure by means of a sensitive enzyme immunoassay (E.I.A.). The mAChR-bearing membrane preparations consisted of native calf or rat forebrain membranes and solubilized calf forebrain membrane preparations. P815 mastocytoma cell membrane preparations devoid of mAChR, as determined by specific radiolabelled dexetimide binding, were used as negative controls.

Polyvinyl chloride (PVC) 96 well microtiter Linbro plates were pre-coated for 1 h with a 10 μg/ml poly-L-lysine solution in H_2O (50 μl/well) at 37°C, and dried before coating with intact membrane preparations. 50 μl aliquots of the membrane suspension (0.1 mg of protein/ml) were introduced into each well of the 96 well PVC microtiter plates and then centrifuged in an Heraeus Labofuge 6000 for 20 min at 3500 r.p.m. The coatings were fixed with 0.25% glutaraldehyde after removal of the supernatant. The plates were then exhaustively washed with PBS and the non-specific sites saturated with a 3% BSA solution.

The coating procedure was slightly different for the application of solubilized membrane preparations. 50 μl aliquots of a solubilized membrane preparation (0.2 mg/ml of protein in PBS) were applied to each well of the PVC 96 well microtiter plates. The plates were washed and non-specific sites saturated as above, after incubation for 2 h at 37°C.

Incubation with the antibody solution (50 μl/well) was carried out for 2 h at 37°C or overnight at 4°C. After incubation the plates were dried and washed several time with 0.1% BSA in PBS. Peroxidase-labeled rabbit anti-mouse IgG antibodies were then added (50 μl/well, 1:500 dilution, Miles or Institut Pasteur Production) and the incubation continued for 1 h at 37°C. After extensive washings with PBS, 200 μl of 40 mM ABTS – 2.5 mM H_2O_2 was added and the coloration which had developed was measured on an Artek Multiscanner at 405 nm.

Selected hybridoma clones were subcloned by limiting dilution (Oi and Herzenberg, 1980) into microwells of 96-well Linbro plates. The screening was again done by E.I.A. tests. Subclones were selected for their reactivity with the different mAChR-bearing preparations. These clones were further propagated in ascites in mice for antibody production and the antibodies were partially purified and concentrated by 50% ammonium sulphate precipitation. The monoclonal antibodies were then examined for reactivity with: (i) purified radioiodinated mAChRs, (ii) digitonin-solubilized active mAChRs, and (iii) Western blots (Towbin *et al.*, 1979) of solubilized calf brain membrane preparation.

Immunoprecipitation of purified raidoiodinated mAChRs

100 μl of a 1:1 protein A-Sepharose CL-4B gel (Pharmacia) suspension in 10 mM phosphate buffer, pH 8, was incubated with 10 μl rabbit anti-mouse IgG in the absence of anti-mAChR antibodies and 50 μl of a solution of purified radioiodinated receptors. The mixture was left overnight at 4°C and then centrifuged for 2 min at 12 000 r.p.m. in an Eppendorf centrifuge to eliminate the non-specifically adsorbed radioiodinated material. 50 μl of the antibody-containing ascitic fluid was then added to the supernatant and the incubation continued for 3 h at room temperature. 100 μl of a protein A-Sepharose Cl-4B suspension treated with 10 μl rabbit anti-mouse IgG antibody was then added and the incubation continued overnight at 4°C. After centrifugation at 12 000 r.p.m. at 4°C, the precipitate was rinsed three times with the phosphate buffer (pH 8). The precipitated gel was finally diluted in denaturating sample buffer before applicaiton on an SDS gel for electrophoresis.

Slab gel SDS-PAGE was performed as described by Laemmli and Favre (1973) with a 4% stacking gel and a 10% separating gel.

Western blots

200 μl (0.5 mg of protein) of the solubilized membrane preparation was treated with 200 μl of denaturating sample buffer before application on a polyacrylamide slab gel and subsequent electrophoresis. The separated components were immediately blotted on nitrocellulose at 100 V for 3 h (electrode distance 10 cm) in a 6 M urea buffer (Towbin *et al.*, 1979). The sheet was saturated with 3% BSA and cut into small strips.

Strips were incubated with a 1:10 dilution of the ascitic fluids or a 2–5 mg/ml solution of the purified antibodies overnight at 4°C. The strips were exhaustively washed with PBS/BSA 0.1% and then incubated with peroxidase-labeled rabbit anti-mouse IgG antibodies (Miles or Institut Pasteur Production, 1:500 dilution) for 2 h at room temperature. After exhaustive washings with PBS, the peroxidase substrate 1 mM H_2O_2 – 3 mM 4-chloro-1-naphthol (Sigma) was added. The reagent was washed away with H_2O as soon as the colored bands appeared.

Immunoprecipitation of digitonin-solubilized mAChR

600 μl of a solubilized calf brain membrane preparation (0.75 mg of solubilized protein) was incubated for 30 min at room temperature with 75 μl of ascitic fluid. The mixture was left overnight at 4°C. After centrifugation at 9000 r.p.m. for 15 min at 4°C, two 200 μl aliquots of the supernatant were put into two plastic tubes. [^{3}H]Dexetimide was added to one tube to a final concentration of 2 nM for the determination of total binding. The other tube was made up to a final concentration of 20 nM [^{3}H]dexetimide plus 2 μM atropine for the determination of non-specific binding. The mixtures were incubated for 20 min at 30°C before application of 300 μl of the incubation mixture on a Sephadex G-50 column (André *et al.*, 1983). The protein-bound dexetimide was eluted in the first 3 ml; elution of the free radioligand started at 3.5 ml.

Immunoabsorption of digitonin-solubilized mAChRs

100 μl ascitic fluid was added to 4 g CNBr activated Sepharose 4B in 16 ml 0.1 M bicarbonate solution (pH 8). After 12 h agitation at 4°C, unreacted sites were saturated with bovine IgG. 250 mg gel, exhaustively washed with PBS, was suspended in 375 μl PBS with 25 μl digitonin-solubilized mAChRs.

After overnight incubation at 4°C and subsequent centrifugation, the mAChR content remaining in the supernatant was measured, as above, by the addition of [^{3}H]dexetimide (20 nM final concentration) and analysis over a Sephadex G-50 column.

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