Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 151-157

MONOSPECIFIC ANTIBODIES AGAINST A SYNTHETIC PEPTIDE PREDICTED FROM THE ALPHA-3 NICOTINIC RECEPTOR cDNA INHIBIT BINDING OF [^{3}H]NICOTINE TO RAT BRAIN NICOTINIC CHOLINERGIC RECEPTOR

T. C. Madhok*, C.C. Chao*, S.G. Matta*, A. Hong+ and B.M. Sharp*

*Endocrine-Neuroscience Laboratory,
Minneapolis Medical Research Foundation
and
Departments of Medicine
Hennepin County Medical Center and the University of Minnesota
Minneapolis, MN 55415

+Custom Peptide Synthesis Department
Applied Biosystems,
Foster City, CA 94404

Received October 11, 1989

SUMMARY: Polyclonal antibodies were raised against a synthetic decapeptide (designated S3) predicted from a segment of the alpha-3 subunit cDNA (amino acid residues 130-139) encoding the rat brain nicotinic cholinergic receptor. This segment was selected because it may be proximate to the nicotine/acetylcholine-binding site of the receptor (1). By radioligand binding assays and sucrose density gradient centrifugation, these monospecific antibodies were shown to inhibit the binding of [^{3}H]nicotine to both the large molecular weight rat brain receptor (240 kDa) and to an SDS-disaggregated nicotine-binding subunit species (80 kDa), in a dose-dependent manner. The neutralizing effect of the anti-S3 antibodies supports the view that this region of the protein is closely related to the agonist binding site.

Polyclonal and monoclonal antibodies as well as molecular cloning have been used to characterize the structure and function of the nAcChR. Using the purified *Torpedo* receptor, Lindstrom and his collaborators have generated anti-peripheral nAcChR monoclonal antibodies which recognize the chick brain nAcChR (2-4). Antibodies against this receptor were then used to identify 51 and 79 kDa subunits of the rat brain nAcChR. Using an anti-idiotypic monoclonal antibody against nicotine antibodies, a rat brain receptor subunit (56 kDa) was also isolated by Langone and his coworkers (5). Patrick and his collaborators (6,7) have utilized molecular cloning to identify cDNAs encoding several different agonist binding subunits (alpha) of the rat brain nAcChR. We have synthesized a peptide from the predicted amino acid sequence (residues 130-139; i.e. I-D-V-T-Y-F-P-F-D-Y) of the alpha-3 cDNA (1). This was used to investigate the nicotine binding domain of the rat brain nAcChR. We report herein the production of polyclonal antibodies against

ABBREVIATION: nAcChR, nicotinic cholinergic receptor.

0006-291X/89 $1.50

this S3 peptide segment, and the capacity of these affinity-purified antibodies to inhibit the binding of [^{3}H]nicotine to the large molecular weight nAcChR species (240 kDa) and to the nicotine-binding subunit (80 kDa).

MATERIALS AND METHODS

Chemicals. L-(-)-[N-Methyl-^{3}H]-Nicotine (67.2 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned.

Synthesis of the decapeptide. S3 decapeptide was synthesized using solid phase methods (8) with the Applied Biosystems Automatic Synthesizer Model 430 A and the purity was assessed by HPLC (0.1% TFA in water/0.09% TFA, 60% CH_3CN, 40% H_20).

Preparation of the rat brain P2 membrane. P2 membrane from rat brain was obtained as described by Wonnacott (9). Briefly, adult rat brains were homogenized in sucrose buffer, pH 7.5, containing 1 mM EDTA, 0.1 mM PMSF and 0.01% (w/v) sodium azide. After the removal of cell debris, nuclei, etc., the high-speed membrane pellet (12,000xg), P2, was isolated and solubilized with 0.5% Triton X-100 in 50 mM phosphate buffer pH 7.5 containing 0.5 mM PMSF, 5 mM EDTA, and 0.02% (w/v) sodium azide.

Immunization of rabbits and purification of monospecific polyclonal antibodies. Rabbits were immunized with the S3 peptide coupled to thyroglobulin using glutaraldehyde (10). High titer sera were precipitated by 40% saturated ammonium sulfate solution followed by ion-exchange chromatography on a DE-52 cellulose column. The IgG fraction was then sequentially purified on thyroglobulin-Sepharose 4B and S3 peptide-Sepharose 6B (11).

Enzyme-linked immunosorbent assay (ELISA). The titer of the affinity-purified antibodies was determined by ELISA as described (11). Briefly, microtiter plates (Dynatech, Alexandria, VA) were coated with antigen (10 ug/ml) overnight at 4°C and then blocked with 3% gelatin (Bio-Rad, Richmond, CA). Following incubation with the primary antibody for 2 hr, a horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG (Bio-Rad) was added for another 30 min. The plate was read on a Multiscan (Flow Laboratories, McLean, VA) at 414 nm, 10 min after adding the substrate solution (0.03% 2,2'-azino-di-3-ethyl-benzthiazoline sulfonate) in 0.1 M sodium citrate, pH 4.0, containing 0.003% H_20_2.

[^{3}H]Nicotine binding assay. [^{3}H]Nicotine binding to rat brain receptors was measured utilizing the method of Wonnacott (9). Briefly, using triplicate samples, solubilized P2 membrane (200-2000 ug) were incubated with [^{3}H]nicotine (20-40 nM) for 40 min at 20°C in the presence or absence of unlabeled (-) nicotine at a final concentration of 1 mM. Subsequently, samples were placed on ice for 30 min, filtered under vacuum through Whatman GF/B filters which had been presoaked for 3 hr at 40°C in 0.33% (v/v) polyethylenimine, and then washed three times with the ice-cold buffer. Specific binding is defined as that component of total binding displaced by 1 mM (-) nicotine. Solubilized P2 membrane was preincubated with IgG fractions for 1 hr at 20°C before assay for [^{3}H]nicotine binding.

Sucrose density gradient centrifugation. The molecular size of the different receptor forms was determined by their sedimentation on a 5-40% or 10-30% (w/v) sucrose gradient in HEPES buffer, pH 7.5, containing 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$ and 1.2 mM $MgSO_4$. Centrifugation was accomplished for 16 hr at 45,000 RPM at 4°C . Fractions, 0.08 ml, were collected and assayed for [^{3}H]nicotine binding.

Preparation of nAcChR subunit species. Following the procedure of Haggerty and Froehner (12), rat P2 membranes were solubilized with 1% SDS for 1 hr at 4°C and dialyzed

against 0.1% cholate in phosphate buffer pH 7.5 for 4 hr at 4°C. A 4 mg aliquot was loaded on a 10-30% sucrose gradient and centrifuged for 16 hr at 45,000 RPM.

RESULTS

Purification of IgG against S3-decapeptide. The titration curve for the binding of anti-S3 IgG to S3 peptide is shown in Figure 1. Affinity-purified antibodies to an irrelevant decapeptide (Anti-IrP), and an ion-exchange purified pre-immune IgG (Pre-IgG) showed no reactivity with S3 peptide at various concentrations. In addition, two irrelevant hexadecapeptides failed to react with anti-S3 IgG (data not shown).

Affinity-purified antibodies inhibit the binding of [3H]nicotine to solubilized rat brain membrane receptor. Since the decapeptide used for immunization represents a segment of the alpha-3 subunit of the brain nAcChR which may be near the nicotine binding pocket, investigations determined whether the anti-S3 peptide IgG could modify the binding of [^{3}H]nicotine to solubilized membrane receptor. As shown in Figure 2, incubation of the solubilized membrane receptor with increasing concentrations of affinity-purified anti-S3 IgG led to a concentration dependent inhibition of [^{3}H]nicotine binding to the P2 membrane. Maximum inhibition was achieved upon preincubation with 180 ug (approximately 5 x 10^{-6}M) of the antibody. The same concentration of IgG from preimmune rabbit serum did not inhibit [^{3}H]nicotine binding to the receptor protein. To further evaluate this finding, solubilized receptor membrane was incubated

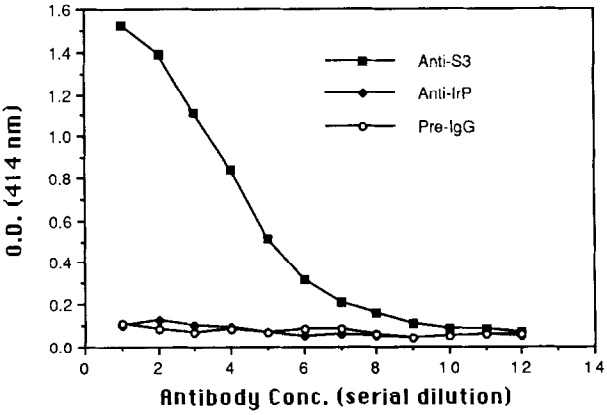

Figure 1. Antigen binding capacity of the affinity-purified anti-S3 IgG. This is the titration curve of the purified anti-S3 IgG against the S3 decapeptide. The starting antibody concentration in well 1 was 15 ug/ml, whereas the concentration in well 12 was 7.3 ng/ml. Anti-IrP and Pre-IgG refer to monospecific antibodies against an irrelevant decapeptide and pre-immune rabbit IgG, respectively.

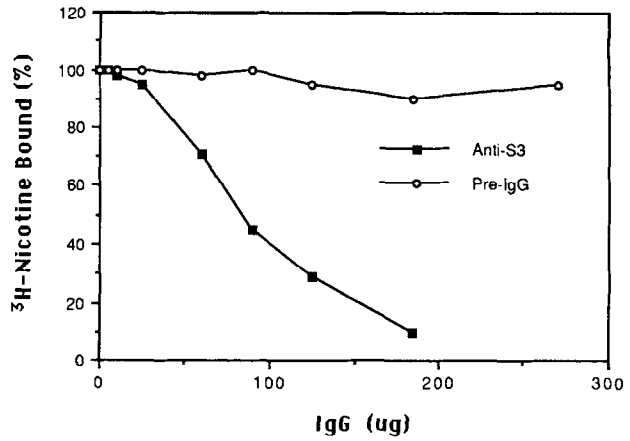

Figure 2. Dose-response curve of [^{3}H]nicotine binding to solubilized P2 membrane. Following pre-incubation with increasing amounts of affinity-purified anti-S3 peptide IgG (1.26-178 ug) or pre-IgG (IgG from pre-immune serum, 5-270 ug), [^{3}H]nicotine binding assay was done.

with the anti-S3 IgG for 1 hr at 20°C and then loaded on a 5-40% sucrose gradient. Fractions collected from each gradient were incubated with [^{3}H]nicotine. An 83% inhibition of [^{3}H]nicotine binding to the large molecular weight receptor (240 kDa) was observed (Figure 3).

To determine whether the inhibitory action of anti-S3 IgG depends on its interaction with the agonist-binding subunit of the nAcChR, additional experiments were performed. An SDS-disaggregated receptor protein was obtained by solubilizing P2 membrane with 1% SDS and

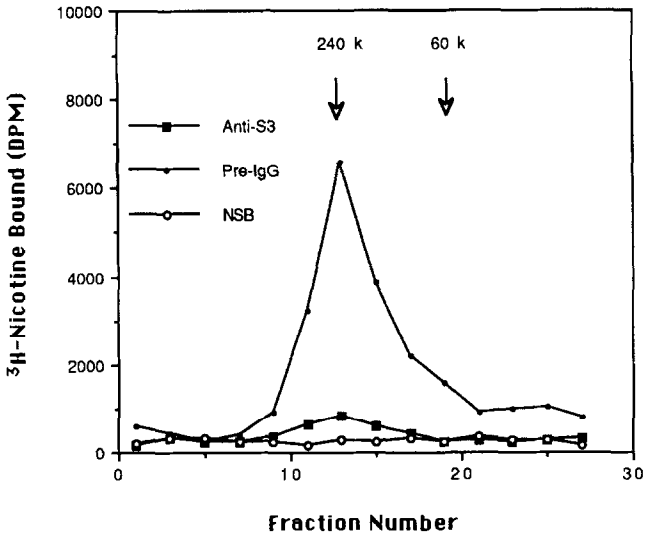

Figure 3. Sucrose density gradient centrifugation profiles. [^{3}H]nicotine binding was done on eluate fractions obtained after incubation of solubilized P2 membrane with anti-S3 peptide IgG versus pre-IgG. NSB is non-specific binding. Protein markers, catalase (240 kDa) and hemoglobin (60 kDa), are indicated by arrows.

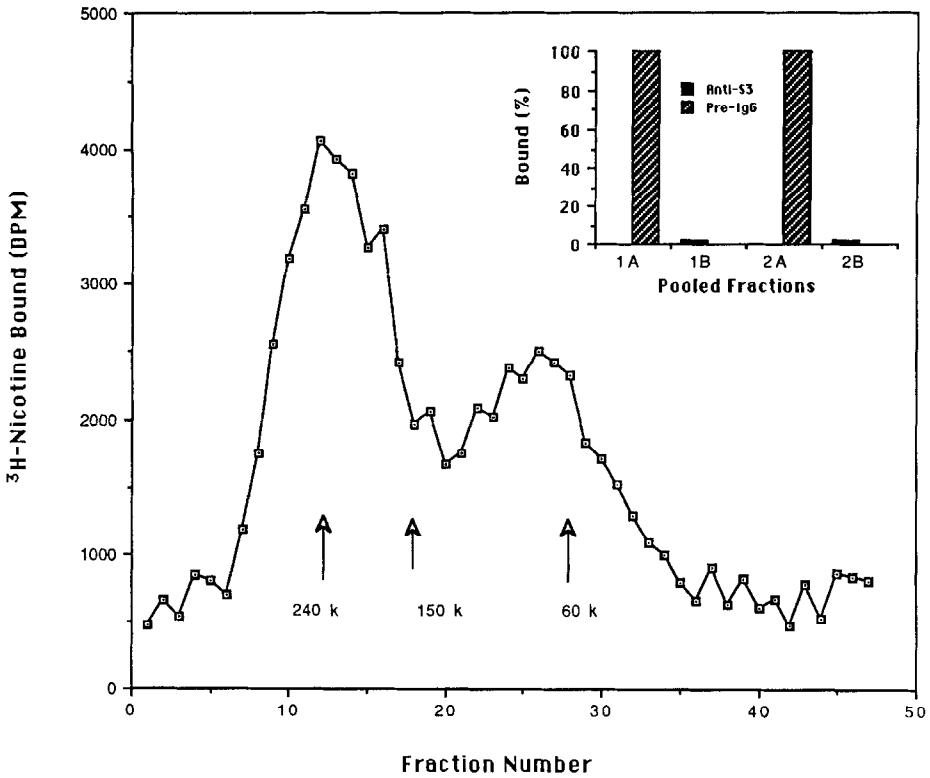

Figure 4. SDS-disaggregation of the large molecular weight nAcChR. The sedimentation profile was obtained after pre-incubation with 1% SDS and dialysis against 0.1% cholate buffer. The protein markers, catalase (240 kDa), alcohol dehydrogenase (150 kDa) and hemoglobin (60 kDa), are indicated by arrows Inhibition of [^{3}H]nicotine binding to the SDS-disaggregated nAcChR subunit is shown in the bar graph insert. Pooled samples, containing fractions 10-15 for the 240 kDa species (bars 1A and 1B) and fractions 23-32 for the 80 kDa (bars 2A and 2B) were incubated with anti-S3 IgG (bars 1B and 2B) or pre-IgG (bars 1A and 2A).

subsequently dialyzing against 0.1% cholate. Figure 4 shows that approximately 40% of the large receptor molecule was converted to a smaller binding subunit (80 kDa) which is in agreement with the reported size of the agonist binding subunit of the rat brain nAcChR (4). Samples from the 240 kDa receptor species (fractions 10-15) versus the 80 kDa binding subunit species (fractions 23-32), were pooled, separately. Each pooled sample was incubated with anti-S3 IgG or preimmune IgG and then assayed for [^{3}H]nicotine binding. Figure 4 (bar graph insert) demonstrates the complete abolition of [^{3}H]nicotine binding to both the intact nAcChR and its subunit species by the anti-S3 IgG compared to the preimmune IgG.

DISCUSSION

The agonist (nicotine/acetylcholine) binding site of both the brain and peripheral nAcChR is not clearly defined. Regarding the peripheral nAcChR (*Torpedo californica*), the

acetylcholine/nicotine and the alpha-bungarotoxin binding sites are both on the same alpha-receptor subunit (13). Regions of the alpha subunit containing disulfide bonds appear to be part of the agonist binding site (13-16). Studies of the alpha-subunit from peripheral nAcChRs have revealed four cysteine residues at positions 128, 142, 192 and 193, and various combinations of these have been reported to form disulfide bridges; among the suggested pairs, Cys-128 to Cys-142 and Cys-192 to Cys-193 are most likely (16). Thus, it has been suggested that the agonist binding site involves sequences which include parts of the region between Cys-128 and Cys-193. In contrast to these studies of the peripheral nAcChR, acetylcholine/nicotine and alpha-bungarotoxin bind to two distinctly different holoproteins in the brain (17). Although disulfide bridges are also involved in the nicotine binding site, no other information is available to specify the domain of the binding site.

From the predicted sequence of the alpha-3 subunit of the rat brain receptor encoded by a cDNA originally cloned from PC12 cells (1), we have synthesized a decapeptide comprising amino acid residues 130-139. We have purified IgG specific to this peptide and have determined the interaction of the antibody with the rat brain nAcChR. Our data showed a dose dependent inhibition by the anti-S3 IgG of [^{3}H]nicotine binding to the large molecular weight species (240 kDa) of the receptor which is thought to represent the native holoprotein (18). Experiments were performed to generate the ligand-binding subunit of the receptor and to study the interaction of the antibody with this subunit. Our data show that the hydrophobic interaction between the rat brain nAcChR subunits can be effectively disrupted with a high concentration of detergents like SDS (12) and that nicotine binding to a subunit species is preserved after dialysis against a cholate buffer. As with the large receptor species, nicotine binding to the subunit species can also be totally inhibited by this monospecific polyclonal antibody. These findings suggest that the S3 region of the rat brain nAcChR is closely related to the nicotine binding site on all of the rat brain alpha subunits since the binding of [^{3}H]nicotine to both the native receptor and the subunit was completely abolished by the anti-S3 IgG.

ACKNOWLEDGMENTS

We would like to thank Jana Lindh for her technical assistance. This work was supported in part by P.H.S. grants DA-04446 (T.C.M.) and DA-04196 (B.M.S.).

REFERENCES

1. Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., and Patrick, J. (1986) Nature 319, 368-374.
2. Lindstrom, J., Tzartos, S., Gullick, W., Hochschwender, S., Swanson, L., Sargent, P., Jacob, M., and Montal, M. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 89-99.
3. Whiting, P.J., and Lindstrom, J.M. (1986) Biochemistry 25, 2082-2093.
4. Whiting, P.J., and Lindstrom, J.M. (1987) Proc. Natl. Acad. Sci. USA 84, 595-599.
5. Abood, L.G., Langone, J.J., Biercke, R., Lu. X., and Banerjee, S. (1987) Proc. Natl. Acad. Sci. USA 84, 6587-6590.
6. Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J., and Heinemann, S. (1987) Cell 48, 965-973.
7. Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E.S., Swanson, L.W., Heinemann, S., and Patrick, J. (1988) Science 240, 330-334.
8. Young, S. (1984) Solid Phase Peptide Synthesis 2nd Ed. Pierce Chem. Co.
9. Wonnacott, S. (1987) Human Toxicol. 6, 343-353.
10. Reichlin, M. (1980) Meth. Enzymol. 70, 159-165.
11. Chao, C.C., Xu, C., Tung, E., Jordan, D., Wang, I., and Wang, A. (1989) Cell Immunol. 120, 430-438.
12. Haggerty, J.G., and Froehner, S.C. (1981) J. Biol. Chem. 256, 8294-8297.
13. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M, Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., and Numa, S. (1982) Nature 299, 793-797.
14. Kao, P.N., Dwork, A.J., Kaldany, R.J., Silver, M.L., Wideman, J., Stein, S., and Karlin, A. (1984) J. Biol. Chem. 259, 1162-1165.
15. Numa, S., Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y, and Kikyotani, S. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 57-70.
16. Mosckovitz, R., and Gershoni, J.M. (1988) J. Biol. Chem. 263, 1017-1022.
17. Schneider, M., Adee, C., Betz, H., and Schmidt, J. (1985) J. Biol. Chem. 260, 14505-14512.
18. Ruchel, B., Walters, D., and Maelicke, A. (1981) FEBS 119, 215-223.