Localisation of the main immunogenic region of the nicotinic acetylcholine receptor

T. Barkas, J.-M. Gabriel, M. Juillerat, A. Kokla+ and S.J. Tzartos+

Département de Biochimie, Université de Genève, Sciences II, 30, Quai Ernest Ansermet, CH 1211 Genève, Switzerland and +*Hellenic Pasteur Institute, 127, Avenue Vassilissis Sofias, 11521 Athens, Greece*

Received 2 December 1985; revised version received 16 December 1985

The nicotinic acetylcholine receptor from *Torpedo marmorata* was digested using papain and the reaction products separated by SDS gel electrophoresis and characterised by immunoblotting using labelled α-bungarotoxin, polyclonal antibodies to synthetic peptides and monoclonal antibodies to the main immunogenic region (MIR). Using this approach, it was possible to show that the MIR is located N-terminal to all or part of peptide 151–169 (peptide P1) of the α-chain and that papain cleaves the α-chain between Asn 141 and peptide P1.

Acetylcholine receptor *Immunogenic region* *Synthetic peptide*

1. INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) is the major autoantigen in the human autoimmune disease, myasthenia gravis (review [1]). Autoantibodies to the receptor can be detected in the sera of about 90% of myasthenic patients [2]. Experimental models of the disease can be induced readily by immunisation with nAChR purified from electric fish, such as the ray, *Torpedo,* or from mammalian muscle (review [1]). In both the natural and experimental systems, the majority of antibodies are directed against a relatively small region of the nAChR consisting of neighbouring epitopes, the main immunogenic region (MIR) [3–6]. The receptor consists of 4 polypeptide chains (α, β, γ, δ), present in the ratio 2:1:1:1 (review [7]). Antibodies to the MIR recognise the α-chain, which also carries the binding site for cholinergic ligands and specific probes, such as α-bungarotoxin. The sequences of all 4 chains of *Torpedo* nAChR have been determined by the use of cloning techniques (review [7]). Based on these data, it was initially proposed that the MIR might be contained in the region of residues 161–166 of the α-chain [8,9]. Using synthetic peptides, we have presented data suggesting that this is unlikely [10], this subsequently being confirmed by other workers [11]. Using proteolytic cleavage and immunoblotting techniques, we are now able to localise the MIR to a region N-terminal to all or part of residues 151–169 of the α-chain.

Abbreviations: PMSF, phenylmethanesulphonyl fluoride; MBTA, (4-(*N*-maleimido)benzyl)trimethylammoniumiodide, PBS phosphate-buffered saline (pH 7.1)

2. MATERIALS AND METHODS

2.1. *Preparation of nAChR and 'trypsinised nAChR'*

The nAChR was purified from the electric organs of *T. marmorata* as described [12], except that the detergent Triton X-100 was replaced by Emulphogene (polyoxyethylene 10 tridecyl ether, Sigma).

'Trypsinised nAChR' was prepared as follows (all manipulations being carried out at 4°C unless stated otherwise). 100 g electric organ were homogenised in 200 ml of 10 mM potassium phosphate buffer containing 10 mM EDTA, 0.4 M NaCl, 0.1 mM PMSF, 0.01% NaN_3, pH 7.4, for two

Published by Elsevier Science Publishers B.V.
00145793/86/$3.50

periods of 1 min using a Virtis 45 homogeniser. The homogenate was centrifuged at 6000 × *g* for 10 min, and the supernatant recentrifuged at 20 000 × *g* for 1 h. The pellet from the second centrifugation was resuspended in 6 ml of 50 mM potassium phosphate buffer containing 0.4 M NaCl, 0.01% NaN_3, pH 7.4 (buffer A) and the protein content determined. The concentration was adjusted to 5 mg/ml with buffer A and 1 mg/ml trypsin (Sigma) added. The suspension was sonicated for 3 periods of 30 s and incubated at 22°C for 30 min. 100 μl of 0.2 M PMSF was added, the suspension resonicated, and then centrifuged at 100 000 × *g* for 1 h. The pellet was extracted with 30 ml of 10 mM potassium phosphate buffer containing 2% Emulphogene, 10 mM EDTA, 0.2 mM PMSF, 0.01% NaN_3, pH 7.4, for 1 h and the extract processed normally by affinity chromatography. The α-chain of the purified material is found to lack the C-terminal 10 kDa unit as determined by direct and competitive radioimmunoassay or immunoblotting using antibodies to two synthetic peptides from this region (unpublished).

2.2. *Synthesis of peptides and preparation of antisera*

Peptides P1 (residues 151–169) and P3 (residues 426–437) of the α-chain were synthesised and used to raise antisera in rabbits as in [10,13].

2.3. *Iodination techniques*

Purified α-bungarotoxin [14] was iodinated to a specific activity of 900 Ci/mmol as described in [15].

Protein A (Sigma) was iodinated to a specific activity of 18 Ci/mg according to [16].

2.4. *Monoclonal antibody production*

The production and characterisation of the monoclonal antibodies used have been described [3–6]. Culture supernatants were concentrated approx. 20-fold by precipitation with ammonium sulphate, and then dialysed against PBS containing 0.05% NaN_3.

2.5. *Proteolytic digestion*

nAChR or trypsinised nAChR at 1 mg/ml in 10 mM potassium phosphate buffer containing 0.5 M NaCl, pH 7.4, was mixed with an equal volume of 0.25 M Tris-HCl buffer containing 1% (w/v) SDS, 20% (v/v) glycerol, pH 6.8, and boiled for 2 min. Papain (type III, Sigma, 1 mg/ml in water) was then added to give an enzyme-substrate ratio of 1:5. Digestion was performed at 25°C for 45 min. The mixture was then boiled for 2 min to stop the reaction. SDS and mercaptoethanol were added to final concentrations of 1 and 2.5%, respectively, and the mixture again boiled. Control samples were treated identically except that papain was omitted.

2.6. *Electrophoresis and blotting methods*

Electrophoresis was performed using the SDS-polyacrylamide system of Laemmli as described in [13]. Typically, 15–20 μg protein was applied per lane of a 12.5% gel and electrophoresis was performed at 40–100 V until the tracking dye was near the bottom of the gel. The gel was then soaked in 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% (v/v) methanol (buffer B) for 20 min. Electrophoretic transfer to 0.45 μm nitrocellulose was carried out in buffer B at 400 mA for 3 h at 4°C. All subsequent operations were performed at 22°C. The nitrocellulose sheet or strips were incubated for 1 h in PBS containing 0.05% Tween 20, pH 7.1 (buffer C), in plastic bags or tubes. Appropriate volumes of antisera or monoclonal antibody samples diluted 1/100 in buffer C were added and incubated for 1 h. Two washing steps of 20 min using buffer C were performed. In the case of monoclonal antibodies, an equal volume of a rabbit anti-rat IgG diluted 1/100 in buffer C was then added, incubated for 1 h and washed as above. Radiolabelled protein A (2 vols with respect to antisera, final concentration 24 ng/ml in buffer C) was added, incubated for 1 h and again washed twice. In the case of α-bungarotoxin, the labelled toxin (2.5 nM in buffer C) was added and incubated for 2.75 h before two washes as above. Dried blots were then exposed at −70°C using Kodak X-Omat S film and one intensifying screen.

2.7. *Concanavalin A absorption*

After digestion, the material was incubated with 0.6 vol. of packed concanavalin A-Sepharose (Pharmacia) for 1 h at 22°C. After centrifugation, the supernatant was adjusted to 1% SDS and 2.5% mercaptoethanol and processed for electrophoresis.

3. RESULTS

3.1. *Specificity of binding*

As shown in fig.1, monoclonal antibodies to the MIR, polyclonal antisera to synthetic peptides P1 and P3, and labelled α-bungarotoxin bound only to the α-chain of the receptor.

3.2. *Papain digestion products*

After papain digestion, antibodies to the MIR bound to a major digestion product of 19 kDa (fig.1). Variable amounts of material at 10–13 kDa, reactive with the anti-MIR antibodies, were also observed. Identical results were obtained with 6 other monoclonal antibodies to the MIR (mAbs 6, 47, 50, 71, 198, 203; not shown). In contrast, α-bungarotoxin and antibodies to peptides P1 and P3 bound to a major digestion product of 27 kDa and to a minor product of approx. 36 kDa (fig.1). Toxin and antibodies to peptide P1 also bound to a minor component of approx. 24 kDa.

3.3. *Relative positions of the peptides in the α- chain*

(i) As noted above, material reactive with antibodies to the C-terminal peptide P3 comigrated with the material reactive with antibodies to P1 (fig.1).

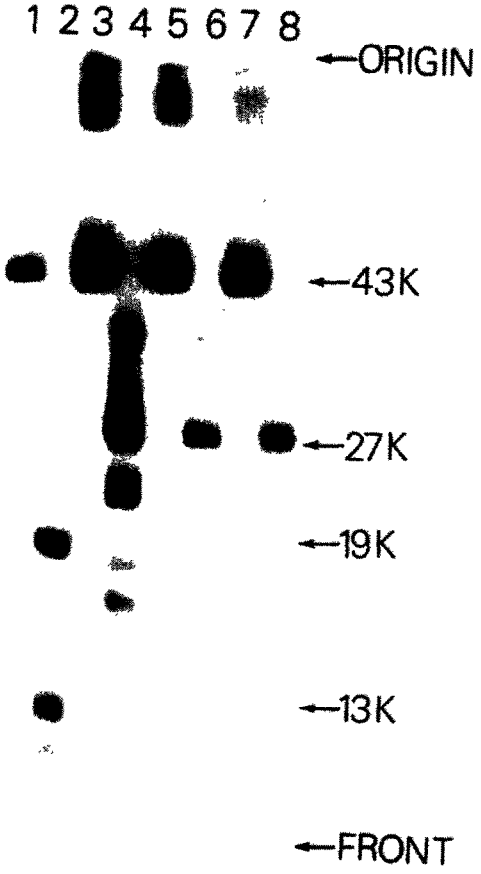

Fig.1. Blots of nAChR and papain-treated nAChR. Digestion, electrophoresis and transfer were performed as described in section 2. Lanes 1, 3, 5 and 7 contain undigested receptor, lanes 2, 4, 6 and 8 papain-digested receptor. Blotting was performed with anti-MIR monoclonal antibody 35 (lanes 1, 2), with antisera to peptide P1 (lanes 3, 4) or peptide P3 (lanes 5, 6) or with labelled α-bungarotoxin (lanes 7, 8).

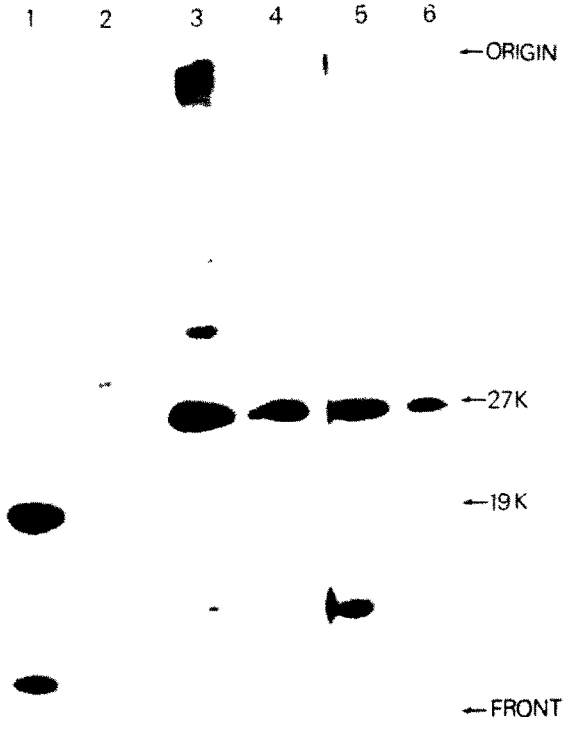

Fig.2. Concanavalin A absorption of papain digestion products. Digestion, electrophoresis and transfer were performed as described in section 2. Lanes 1, 3 and 5 contain non-absorbed digestion products, lanes 2, 4 and 6 the supernatants from the concanavalin A absorption stage. Strips were blotted with anti-MIR antibody 35 (lanes 1, 2), antiserum to peptide P1 (lanes 3, 4) and antiserum to peptide P3 (lanes 5, 6).

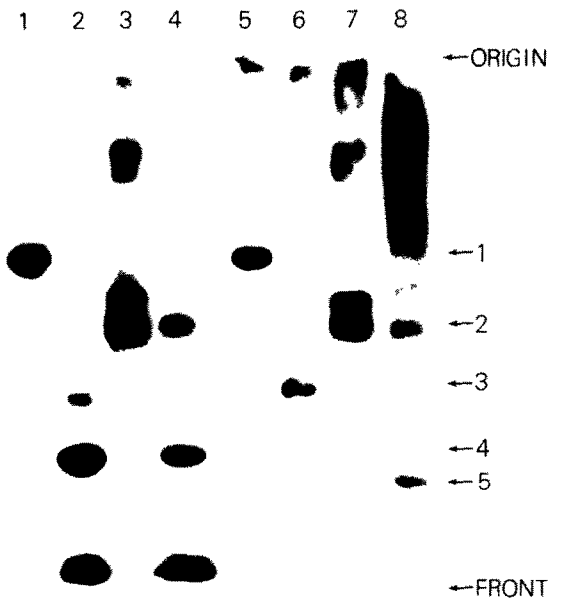

Fig.3. Papain digestion of trypsinised receptor. Digestion, electrophoresis and transfer were performed as described in section 2. The samples applied were untreated nAChR (lanes 1, 5), papain-digested nAChR (lanes 2, 6), untreated trypsinised nAChR (lanes 3, 7) and papain-treated trypsinised receptor (lanes 4, 8). Lanes 1–4 were developed using anti-MIR monoclonal antibody 35, lanes 5–8 using antipeptide antiserum specific for peptide P1. Band 1 is the α-chain of the nAChR. Band 2, a doublet, is the α-chain of trypsinised nAChR.

(ii) Absorption with concanavalin A, which binds to carbohydrate linked to Asn 141 of the α-chain, removed completely the peptide containing the MIR, but not the P1 or P3 containing peptide (fig.2). The material reactive with toxin was also not removed (not shown).

(iii) Digestion of trypsinised nAChR, which lacks the C-terminal 10 kDa unit, produced an identical product of 19 kDa reactive with anti-MIR antibodies (band 4, fig.3) whereas the 27 kDa fragment reactive with anti-P1 antibodies (band 3, fig.3) was replaced by a fragment of 17 kDa (band 5, fig.3).

4. DISCUSSION

Previous experiments [17] have shown that papain cleavage of purified α-chain generates a fragment of 27 kDa which is labelled by the site-directed affinity ligand MBTA and fragments of 17 and 10 kDa which contain carbohydrate and bind antibodies to the MIR. Similarly, Tzartos and Changeux [18,19] have shown that, whereas renatured whole chain can bind both toxin and some antibodies to the MIR, the 27 kDa fragment, after renaturation, could bind only toxin. However, further characterisation of these peptides was not possible because of a lack of suitable markers. The ability to synthesise peptides chosen from the published sequence now allows us to raise antibodies to specific sites as markers to characterise these products.

Using polyclonal antisera to synthetic defined peptides and monoclonal antibodies to the MIR, we have been able to show that the MIR and concanavalin A reactive carbohydrate reside on the same peptide (fig.2) and that the α-bungarotoxin-binding site is carried on a larger peptide containing peptides P1 (151–169) and the C-terminal peptide P3 (425–437). As only carbohydrate groups linked to asparagine are known to react with concanavalin A [20] and as the only suitable asparagine group of the α-chain is Asn 141, it follows that this residue is carried on the smaller 19 kDa peptide. Since residues 151–437 are present in the 27 kDa fragment and residue 141 in the 19 kDa fragment, the MIR can be localised N-terminal to peptide P1. This is confirmed by papain cleavage of trypsinised nAChR (lacking the C-terminal 10 kDa unit). As expected, the N-terminal MIR containing peptide still has a molecular mass of 19 kDa, but the C-terminal peptide containing P1 changes its molecular mass by approx. 10 kDa. The MIR can thus be localised N-terminal to all or part of peptide P1 (151–169) and papain cleavage occurs between Asn 141 and peptide P1.

ACKNOWLEDGEMENTS

This work was supported by grant nos 3.450.0.83 and 3.145.0.85 from the Swiss National Foundation for Scientific Research and by the Greek General Secretariat of Research and Technology. The secretarial assistance of Mrs M. Pittet is gratefully acknowledged.

REFERENCES

[1] Vincent, A. (1980) Physiol. Rev. 60, 756–825.

[2] Lindstrom, J.M., Seybold, M.E., Lennon, V.A., Whittingham, S. and Duane, D.D. (1976) Neurology 26, 1054–1059.

[3] Tzartos, S.J. and Lindstrom, J.M. (1980) Proc. Natl. Acad. Sci. USA 77, 755–759.

[4] Tzartos, S.J., Rand, D.E., Einarson, B.L. and Lindstrom, J.M. (1981) J. Biol. Chem. 256, 8635–8645.

[5] Tzartos, S.J., Seybold, M.E. and Lindstrom, J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 188–192.

[6] Tzartos, S.J., Langeberg, L., Hochschwender, S. and Lindstrom, J.M. (1983) FEBS Lett. 158, 116–118.

[7] Changeux, J.-P., Devillers-Thiery, A. and Chemoulli, P. (1984) Science 225, 1335–1345.

[8] Noda, M., Takashashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. and Numa, S. (1982) Nature 299, 793–797.

[9] Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983) Nature 302, 528–532.

[10] Juillerat, M.A., Barkas, T. and Tzartos, S.J. (1984) FEBS Lett. 168, 143–148.

[11] Lindstrom, J., Criado, M., Hochschwender, S., Fox, J.L. and Sarin, V. (1984) Nature 311, 573–575.

[12] Barkas, T., Gairns, J.McP., Kerr, H.J., Coggins, J.R. and Simpson, J.A. (1982) Eur. J. Immunol. 12, 757–761.

[13] Barkas, T., Juillerat, M., Kistler, J., Schwendimann, B. and Moody, J. (1984) Eur. J. Biochem. 143, 309–314.

[14] Monnier, V.M. and Fulpius, B.W. (1977) Clin. Exp. Immunol. 29, 16–22.

[15] Barkas, T. and Fulpius, B.W. (1984) Biochem. Soc. Trans. 12, 813–814.

[16] Barkas, T. (1981) J. Clin. Lab. Immunol. 5, 59–65.

[17] Gullick, W.J., Tzartos, S. and Lindstrom, J. (1981) Biochemistry 20, 2173–2180.

[18] Tzartos, S.J. and Changeux, J.-P. (1983) EMBO J. 2, 381–387.

[19] Tzartos, S.J. and Changeux, J.-P. (1984) J. Biol. Chem. 259, 11512–11519.

[20] Krusius, T. (1976) FEBS Lett. 66, 86–89.