Methyllycaconitine: a selective probe for neuronal α-bungarotoxin binding sites

J.M. Ward*, V.B. Cockcroft, G.G. Lunt, F.S. Smillie and S. Wonnacott

Department of Biochemistry, University of Bath, Bath BA2 7AY, UK

Received 20 June 1990

The ability of methyllycaconitine (MLA) to inhibit the binding of [^{125}I]α-bungarotoxin to rat brain membranes, frog and human muscle extracts and the human muscle cell line TE671 has been measured. MLA showed a markedly higher affinity for the rat brain site (K_i 1.4×10^{-9} M) than for the muscle receptors (K_i 10^{-5}–10^{-6} M). Structure modelling techniques were used to fit the structure of MLA to a nicotinic pharmacophore model. MLA is the first low molecular weight ligand to be shown to discriminate between muscle nicotinic receptors and their α-bungarotoxin-binding counterpart in the brain, and as such may be a useful structural probe for pursuing the structural and functional properties of the neuronal protein.

Methyllycaconitine; Nicotinic receptor; Brain α-bungarotoxin binding site; Nicotinic pharmacophore

1. INTRODUCTION

The alkaloid *N*-methyllycaconitine (MLA; Fig. 1) is the principal toxic component of the seeds of *Delphinium brownii*. Historically, the seeds from this plant have been recognized for their insecticidal property, which is believed to arise from the potent antagonism of insect nicotinic acetylcholine receptors (nAChR) by MLA [1]. MLA is a potent competitor of [^{125}I]α-bungarotoxin binding to nAChR in flyheads [1], locust ganglia [2] and cockroach nerve cord [3], with K_i values in the nanomolar range. Complete blockade of nicotinic responses from a cockroach motorneurone was observed at 10^{-6} M MLA; the ED_{50} for this antagonism was about 10^{-7} M [3], which is comparable to the sensitivity to MLA of acetylcholine responses in the nematode *Ascaris* [4]. This potency contrasts with the weaker antagonism by MLA of mammalian nAChR at the rat neuromuscular junction (ED_{50} 2×10^{-6} M) [5].

In a previous study [2] we compared the potencies of MLA in competition binding assays for locust and rat neuronal nAChR, and reported that the [^{125}I]α-bungarotoxin binding site in mammalian brain was 3 orders of magnitude more sensitive to MLA than was the putative nAChR labelled by [^{3}H]nicotine in the same tissue. Apart from the snake α-toxins, this is the only example of a cholinergic compound that is more potent at the α-bungarotoxin site than the [^{3}H]nicotine site. Thus the insect and rat brain [^{125}I]α-bungarotoxin binding sites share a common sensitivity to MLA. In view of the current uncertainty about the physiological status of [^{125}I]α-bungarotoxin binding sites in the mammalian CNS (see [6,7]), a pharmacological probe showing selectivity for this site would be very useful. Although the α-bungarotoxin binding component in vertebrate brain shows homology with nAChR at the protein [8] and gene [7] level, there is little direct evidence that this protein has any role in synaptic transmission in the brain, despite its clear nicotinic pharmacology in binding experiments. Exceptions include the chick optic tectum [8] and cerebellar interneurones [9], where α-bungarotoxin has been shown to antagonize nicotinic responses. In insects, however, the neuronal α-bungarotoxin binding protein is well documented as a functional nAChR [10]. The potency of MLA for neuronal α-bungarotoxin sites, both mammalian and invertebrate, has prompted us to further assess its specificity by comparing its potencies in competition binding assays for nAChR in vertebrate muscle preparations and to consider the molecular basis for its pharmacological specificity.

2. MATERIALS AND METHODS

2.1. *Materials*

Na ^{125}I was purchased from Amersham International (Aylesbury, UK). α-Bungarotoxin was purchased from Sigma (Poole, UK) and iodinated to a specific activity of 700 Ci/mmol as previously described [11]. Tissue culture reagents were obtained from Flow Laboratories, Irvine, Ayrshire, UK or Gibco Ltd., Uxbridge Middlesex, UK. All sterile plastic ware was supplied by Nunc Gibco Ltd. TE671 cells were kindly provided by Professor J. Newsom-Davis (Institute for Molecular Neuroscience, John Radcliffe Hospital, Oxford, UK) and were cultured in monolayers in 80 cm^3 flasks, essentially as described

Correspondence address: S. Wonnacott, Department of Biochemistry, University of Bath, Bath BA2 7AY, UK

**Present address:* Department of Anesthesiology, Harvard Medical School, Shriners Burns Institute, Boston, MA, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

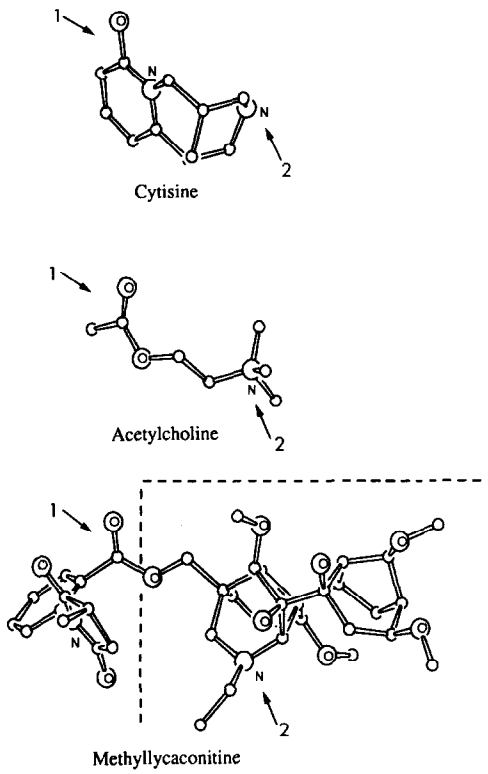

Fig. 1. Structures of MLA, acetylcholine and cystisine. Hydrogen atoms are not shown. Unlabelled atoms are carbon. Arrows indicate the key atoms implicated in the pharmacophore model of nicotinic cholinergic ligand binding [20]. The dashed box encloses the parent structure, lycotonine. The *N*-phenyl-succinimide side chain of MLA is on the left.

by Syapin et al. [12] with modifications [13]. MLA (citrate salt) was synthesized by Professor M.H. Benn (Department of Chemistry, University of Calgary, Alberta, Canada).

2.2. *Competition binding assays*

Rat brain P2 membranes, frog muscle extract and human muscle extract were prepared and assayed for [^{125}I]α-bungarotoxin binding as previously described [2,14,15]. TE671 cells were harvested and replated in fresh growth medium in 24-well plates at a cell density of 5×10^5 cells/ml, essentially as previously described [12,13]. Triplicate wells were incubated with serial dilutions of MLA and [^{125}I]α-bungarotoxin (final concentration 2.0 nM) for 90 min at 37°C. Non-speficic binding was determined in the presence of 1.0 mM D-tubocurarine. After incubation, cells were washed and extracted in 0.1 M NaOH for determination of radioactivity.

2.3. *Structure modelling*

The programs Discover and Insight from Biosym Inc. were used to construct and view structures on an Evans and Sutherland PS 300 picture system. Energy calculations were performed using the valence force-field with the potential parameters of Dauber-Osguthorpe et al. [16]. MLA was constructed using the X-ray structure of aconitine [17], and the stereochemistry of MLA as reported by Jennings et al. [1]. The 5- and 6-membered rings of the *N*-phenylsuccinimide moiety of MLA were set orthogonal, based on the X-ray structure of *N*-*p*-bromophenyl-succinimide. The rigid nicotinic agonist, cystisine, was used as a template in the establishment of a nicotinic cholinergic pharmacophore. Site-points used to overlay MLA and acetylcholine onto cystisine were: the nitrogen atom of the ammonium group; the carbon atom of the carbonyl group; and the oxygen atom of the carbonyl group. Acetylcholine and MLA were template-fitted onto cystisine using a forcing constant of 25 kcal/mol [18].

3. RESULTS AND DISCUSSION

Competition binding assays with MLA were carried out on rat brain membranes, frog and human muscle extracts and the human rhabdomyosarcoma cell line TE671 (Fig. 2). Inhibition constants (the concentration of MLA that inhibits [^{125}I]α-bungarotoxin binding by 50%) are given in Table I. In agreement with our previous findings [2], MLA was a very potent inhibitor of [^{125}I]α-bungarotoxin binding to rat brain membranes. However, it was more than 3 orders of magnitude weaker in competing for the muscle nAChR in each of the preparations tested. Notably, human muscle extract, prepared from amputated calf muscle [15], and the TE671 cell line displayed similar K_i values (approximately 10^{-5} M) for MLA. The sensitivity of muscle nAChR to MLA is very comparable to that previously derived for *Torpedo* NaChR and brain [^{3}H]nicotine binding sites [2] (see Table I). At 10^{-4} M, MLA had no muscarinic potency, indicated by its failure to inhibit [^{3}H]quinuclidinyl benzilate binding to rat brain membranes (data not shown).

The unique discrimination by MLA in favour of neuronal versus muscle α-bungarotoxin binding sites raises the issue of the structural features that underlie the recognition of this compound; the definition of such features would aid our understanding of the molecular nature of the nicotinic receptor recognition site. The parent structure, lycoctonine [1] (see Fig. 1), is without nicotinic potency; indeed it is closely related to aconitine which interacts with sodium channels at the site characterized by batrachotoxin [19]. The introduction of an ester linkage in the formation of MLA provides a carbonyl oxygen (Fig. 1, arrow 1), that is likely to be a key moiety in conferring cholinergic nicotinic ac-

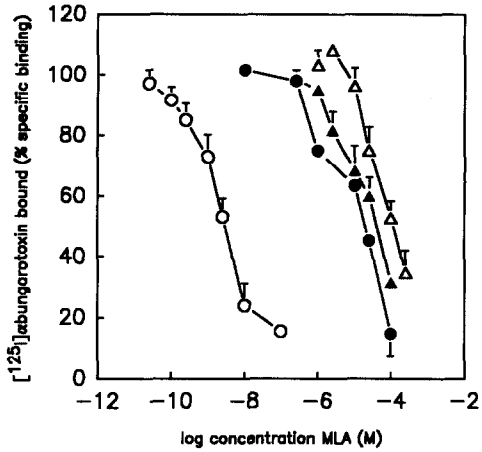

Fig. 2. Competition curves for MLA in various nAChR preparations. Rat brain membranes (○—○), detergent extracts of frog (●—●) and human (▲—▲) muscle, and culture dishes of TE671 cells (△—△) were incubated with serial dilutions of MLA and 2 nM [^{125}I]α-bungarotoxin. Non-specific binding was determined in the presence of unlabelled α-bungarotoxin. Values are the means of at least 3 separate assays with SEM indicated by the vertical bars.

Table I

Inhibition of radioligand binding to nAChR preparations by MLA

Preparation	Ligand	K_i (M) for MLA
Frog muscle extract	[^{125}I]α-Bgt	$1.0 \pm 0.2 \times 10^{-5}$
Human muscle extract	[^{125}]α-Bgt	$7.8 \pm 2.0 \times 10^{-6}$
TE671 cells	[^{125}I]α-Bgt	$6.3 \pm 1.4 \times 10^{-5}$
Rat brain P2 membranes	[^{125}I]α-Bgt	$1.4 \pm 0.2 \times 10^{-9}$
Rat brain P2 membranes	[^{3}H]nicotine	$3.7 \pm 0.7 \times 10^{-6}$*
Torpedo purified nAChR	[^{125}I]α-Bgt	$1.1 \pm 0.6 \times 10^{-6}$*
Locust ganglion	[^{125}I]α-Bgt	1.8×10^{-8}*

Competition assays weer performed using 2 nM [^{125}I]α-Bgt. IC_{50} values were derived from linear transformations of dose-response curves; K_i values were derived from IC_{50} values [26], assuming K_d values for [^{125}I]α-Bgt of 1.8 nM (frog muscle [14]), 0.5 nM (human muscle [15]), 2.0 nM (TE671 cells; Ward, unpublished observations), 1.5 nM (brain [2]). Values are the means ± SE for at least 3 independent determinations. *Data from [2] for comparison.

tivity on this structure. This electronegative centre is a feature of nicotinic ligands, and is considered to be involved in hydrogen bonding with key residues at the recognition site [20].

In addition, this pharmacophore model [20] of nicotinic ligands includes an electrostatic interaction between the quaternary nitrogen of acetylcholine and the receptor binding site. Using the rigid agonist cytisine as a definitive template, acetylcholine and MLA were superimposed to generate the conformations illustrated in Fig. 1 (see Materials and Methods). Thus we can identify the nitrogen atom in MLA for similar electrostatic interaction (Fig. 1; arrow 2).

How can we account for the marked preference shown by MLA for neuronal α-bungarotoxin binding sites compared with other members of the nicotinic receptor family of proteins? The precise spatial relationship between the key residues contributing to the receptor binding site [21,22] may vary among receptor subtypes and hence could influence the affinity of binding of MLA. Sequence analysis of the two α-bungarotoxin binding proteins recently cloned from chick brain [7] shows them to have lower homology with other avian nicotinic receptor subunits (less than 50% in every case [7]) than there is between neuronal and muscle nicotinic receptor subunits. That is to say, within the same species the enigmatic brain α-bungarotoxin binding protein is less similar to the muscle nicotinic receptor, which also binds α-bungarotoxin

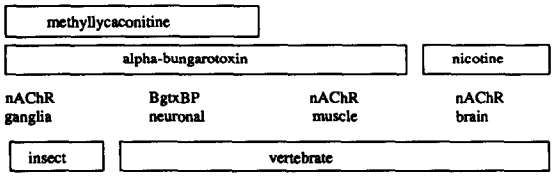

Fig. 3. Classification of nicotinic receptors based on their high affinity binding of MLA, α-bungarotoxin and nicotine.

(see Fig. 3) than are the neuronal nicotinic receptors that do not recognize this snake toxin. However, comparison of the chick brain α-bungarotoxin binding protein sequences with that of an α-subunit cloned from locust ganglion [23] reveals considerably greater homology, especially in the rather variable extracellular domain. In particular, there is high homology in the sequences flanking the vicinal cysteines (Cys 192 and 193 *Torpedo* numbering) that are implicated in the nicotinic ligand binding site [24]. Whereas the oxygen-rich bulk of the lycoctonine portion of MLA (Fig. 1) might be expected to face into the solvent around the binding site, the *N*-phenylsuccinimide side chain is topologically equivalent to the reactive group in ligands that label Cys 192 and 193 [24]. Thus this side chain may be accommodated in this region of the protein, the amino acid sequence of which will influence the relative binding affinities for MLA. The homology between chick brain α-bungarotoxin binding proteins and locust nAChR is likely to underlie the high affinity binding of MLA shared by these proteins (Table I).

The possible relationships in receptor structure that are reflected in the pharmacological similarities are outlined in Fig. 3. It should be noted however that the pharmacological discrimination is not absolute; both muscle nAChR and the high affinity nicotine binding site in brain show a low affinity for MLA (Table I), and all the nicotinic proteins, by definition, recognize nicotine to some extent. Indeed we have recently shown that micromolar concentrations of MLA antagonize functional responses of neuronal nAChR that are insensitive to α-bungarotoxin [25].

We suggest that MLA is a potentially useful probe in the dissection of subtypes of nAChRs. Synthesis of analogues of MLA may well provide more definitive answers to some of the proposals made here and may help in defining more precisely the site(s) of ligand recognition in this important family or neuronal receptor proteins.

Acknowledgements: This study was supported financially by R.J. Reynolds Tobacco Co. (S.W. and G.G.L.), MRC Project Grant G8722675N (S.W.) and a collaborative SERC project grant with Shell Research (G.G.L., V.B.C.). J.M.W. was a recipient of an SERC Postgraduate Training Award. We are indebted to Professor Michael Benn for providing the MLA.

REFERENCES

[1] Jennings, K.R., Brown, D.G. and Wright, D.P. (1986) Experientia 42, 611–613.
[2] MacAllan, D.R.E., Lunt, G.G., Wonnacott, S., Swanson, K.L., Rapoport, H. and Albuquerque, E.X. (1988) FEBS Lett. 226, 357–363.
[3] Sattelle, D.B., Pinnock, R.D. and Lummis, S.C.R. (1989) J. Exp. Biol. 142, 215–224.
[4] Colquhoun, L., Gallagher, T., Holden-dye, L. and Walker, R.J. (1990) Neurosci. Lett. S38, S113.
[5] Nambi Aiyar, V., Benn, M.H., Hanna, T., Jacyno, J., Roth, S.H. and Wilkens, J.L. (1979) Experientia 35, 1367–1368.

[6] Wonnacott, S. (1990) in: Nicotine Psychopharmacology: Molecular, Cellular and Behaviour Aspects, (S. Wonnacott, M.A.H. Russel and I.P. Stolerman, eds) Oxford University Press, pp. 226–277.
[7] Schoepfer, R., Conroy, W.G., Whiting, P., Gore, M. and Lindstrom, J. (1990) Neuron (in press).
[8] Conti-Tronconi, B.M., Dunn, S.M.J., Barnard, E.A., Dolly, J.O., Lai, F.A., Ray, N. and Raferty, M.A. (1985) Proc. Natl. Acad. Sci. USA, 82, 5208–5212.
[9] De la Garza, R., McGuire, T.J., Freedman, R. and Hoffer, B.J. (1987) Neuroscience 23, 887–891.
[10] Breer, H. and Sattelle, D.B. (1987) J. Insect Physiol. 33, 771–790.
[11] Wonnacott, S., Harrison, R. and Lunt, G.G. (1982) Brain Res. 231, 365–377.
[12] Syapin, P.J., Salvaterra, P.M. and Engelhardt, J.K. (1982) Brain Res. 231, 365–377.
[13] Walker, R.J., Vincent, A. and Newsom-Davis, J. (1988) J. Neuroimmunol. 19, 149–157.
[14] Rozental, R., Aracava, Y., Scoble, G.T., Swanson, K.L., Wonnacott, S. and Albuquerque, E.X. (1989) J. Pharmacol. Exp. Ther. 251, 395–404.
[15] Stephenson, F.A., Harrison, R. and Lunt, G.G. (1981) Eur. J. Biochem. 115, 91–97.
[16] Dauber-Osguthorpe, P., Roberts, V.A., Osguthorpe, D.J., Wolff, J., Genest, M., Hagler, A.T. (1988) Proteins: Struct. Funct. Genet. 4, 31–47.
[17] Codding, P.W. (1982) Actga Crystallogr., Sect. B 38, 2519.
[18] Struthers, R.S., Rivier, J. and Hagler, A.T. (1984) in: Conformationally Directed Drug Design: Peptides and Nucleic Acids as Templates or Targets (J.A. Vida and M. Gordon, eds), American Chemical Society, Washington DC, pp. 239–261.
[19] Catterall, W. (1980) Annu. Rev. Pharmacol. Toxicol. 20, 15–43.
[20] Sheridan, R.P., Nilakantan, R., Dixon, J.S., Venkataraghavan, R. (1986) J. Med. Chem. 29, 899–906.
[21] Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chretien, M. and Changeux, J.-P. (1988) Biochemistry 27, 2346–2357.
[22] Cockcroft, V.B., Osguthorpe, D., Barnard, E.A. and Lunt, G.G. (1990) Proteins: Struct. Funct. Genet. (submitted).
[23] Marshall, J. (1989) PhD. Thesis, University of Cambridge.
[24] Kao, P.N. and Karlin, A. (1986) J. Biol. Chem. 261, 8085–8088.
[25] Wonnacott, S., Drasdo, A. and Caulfield, M. (1990) Soc. Neurosci. Abstr.