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Methyllycaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems

David R.E. Macallan, George G. Lunt, Susan Wonnacott, Karen L. Swanson[+], Henry Rapoport* and Edson X. Albuquerque[+]

*Department of Biochemistry, University of Bath, Bath BA2 7AY, England, *Department of Chemistry, University of California, Berkeley, CA 94720* and [+] *Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, MD 21201, USA*

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Specific high-affinity binding sites for ^{125}I-α-bungarotoxin and (−)-[^{3}H]nicotine have been measured in rat brain and locust (*Schistocerca gregaria*) ganglia. The binding sites for ^{125}I-α-bungarotoxin had similar K_d values of 1.5×10^{-9} and 0.8×10^{-9} M for rat and locust preparations, respectively; the corresponding values for the (−)-[^{3}H]nicotine-binding site were 9.3×10^{-9} and 1.7×10^{-7} M. Methyllycaconitine (MLA) potently inhibited ^{125}I-α-bungarotoxin binding in both rat and locust. MLA was a less effective inhibitor of (−)-[^{3}H]nicotine binding whereas (+)-anatoxin-a was a very potent inhibitor at this site in the rat but not in the locust. These data suggest that (+)-anatoxin-a is a useful probe for the high-affinity nicotine-binding receptor in vertebrate brain, whereas MLA is a preferential probe for the subclass of receptor that binds α-bungarotoxin.

α-Bungarotoxin; Nicotine; Methyllycaconitine; Anatoxin-a; Nicotinic receptor; (Rat brain, Locust ganglion)

1. INTRODUCTION

There is a growing body of evidence supporting the idea that there are nicotinic acetylcholine receptors (nAChR) in vertebrate brain which are genetically related to but pharmacologically distinct from the well-characterized peripheral nAChR [1]. It is also clear that there is heterogeneity of such nAChRs within the brain; for example, a distinction can be made between proteins that interact strongly with α-bungarotoxin and those that show high-affinity (−)-nicotine binding [2,3]. Such pharmacological distinctions are paralleled in molecular genetic studies where evidence is emerging that neurones may have families of related genes coding for nAChRs [4]. In insects, cholinergic transmission is confined to the central nervous system and most published work has been based on the assumption that α-bungarotoxin binding defines this receptor. The pharmacological properties of the insect nAChR, however, indicate a specificity that is not identical to that of the vertebrate [5]. In fact, there are limited structural data available for insect receptors and controversy exists regarding the numbers and types of subunits present [6].

We have compared the binding of ^{125}I-α-bungarotoxin and (−)-[^{3}H]nicotine to rat and

Correspondence address: E.X. Albuquerque, Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, MD 21201, USA

Abbreviations: nAChR, nicotinic acetylcholine receptor; MLA, methyllycaconitine

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locust neuronal tissue and have characterized the pharmacology of this binding using several nicotinic ligands. In particular, we have made use of two natural toxins, methyllycaconitine (MLA) and (+)-anatoxin-a. MLA, found in the seeds of the plant *Delphinium brownii*, is a potent insecticide [7] but is only moderately effective as an antagonist at the mammalian neuromuscular junction [8]. (+)-Anatoxin-a, a product of the freshwater alga *Anabaena flos-aquae*, is a potent agonist at the vertebrate neuromuscular junction and at the central nervous system and closely resembles acetylcholine in its channel-activating properties [9–11].

2. MATERIALS AND METHODS

2.1. *Materials*

Adult locusts (*Schistocerca gregaria*) were purchased from Cambridge Biosciences (Cambridge, England). *Torpedo* nAChR was prepared as described [12]. (−)-[*N-methyl*-^{3}H]Nicotine (78 Ci/mmol) and Na^{125}I were purchased from Amersham International (Amersham, England). α-Bungarotoxin was obtained from Sigma (Poole, England) and was iodinated to a specific activity of 700 Ci/mmol [12]. Both (+)- and (−)-anatoxin-a were synthesized by one of us (H.R.). MLA (citrate salt) was a gift from Dr M.H. Benn (Department of Chemistry, University of Calgary, Alberta). (+)-Nicotine di-(−)-tartrate was provided by Dr Barlow (Department of Pharmacology, University of Bristol, Bristol, England) and dihydro-β-erythroidine was supplied by Dr R.G. Benfield (Merck Sharp & Dohme, Hoddesdon, England). Mecamylamine was provided by Dr M. Caulfield (Glaxo, England) and pentolinium and pempidine were supplied by May & Baker (Dagenham, England). 1,1-Dimethyl-4-piperazinium (DMPP) was purchased from Aldrich. Other drugs were purchased from Sigma. All drug solutions were prepared freshly on the day of assay with the exception of α-bungarotoxin and (+)- and (−)-anatoxin-a which were stored frozen as aqueous stock solutions at concentrations of 2.5×10^{-4}, 1×10^{-4} and 5×10^{-2} M, respectively.

2.2. *Preparation of rat brain P2 membrane fraction*

A P2 membrane fraction was prepared from whole brains of adult, male Wistar rats (minus the cerebellum). The tissue was homogenized (10%, w/v) in 0.32 M sucrose solution, pH 7.4, containing 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride and 0.01% (w/v) sodium azide and the suspension was centrifuged at $1000 \times g$ for 10 min. The supernatant was decanted and retained on ice. The pellet was resuspended in 0.32 M sucrose (5 ml/g original wet wt) and recentrifuged. The supernatants were combined and centrifuged at $12000 \times g$ for 30 min to give a P2 pellet. This was resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing protease inhibitors as above, to give a final volume of 2.5 ml/g original wet wt and washed twice by centrifugation and resuspension.

Table 1

Characteristics of radioligand-binding sites in rat brain and locust ganglion P_2 membrane preparations

	(−)-[^{3}H]Nicotine		^{125}I-α-Bungarotoxin	
	Rat brain	Locust ganglia	Rat brain	Locust ganglia
K_d (nM)	9 ± 3	130 ± 3	1.5 ± 0.7	0.8[a]
B_{max} (pmol/g protein)	108 ± 18	4055 ± 403	63 ± 17	1200[a]

[a] Single experiment carried out in triplicate

Data are from linear regression analyses of Scatchard plots and are means ± range or SE of 2 or 4 independent determinations carried out in triplicate

2.3. *Preparation of locust ganglion P2 membrane fraction*

2-week old adult locusts were used for all experiments. A membrane fraction was prepared from supraesophageal ganglia exactly as in [13].

2.4. *Ligand-binding assays*

(−)-[^{3}H]Nicotine binding to P2 membranes was carried out similarly using rat and locust tissues, but in the former case the membrane suspension was first diluted 5-fold in 50 mM potassium phosphate buffer, pH 7.4. Tissue samples (0.25 ml) were incubated with (−)-[^{3}H]nicotine (10 nM for rat and 50 nM for locust unless otherwise stated) for 30 min at 20°C in the presence and absence of excess unlabelled nicotine (10^{-3} M) to determine nonspecific binding. In competition assays, tissue samples were preincubated with serial dilutions of drug for 10 min at 20°C prior to addition of radioligand. The samples were chilled on ice, diluted with 2 ml ice-cold phosphate-buffered saline (10 mM potassium phosphate, pH 7.4, containing 140 mM NaCl) and rapidly filtered under vacuum on Whatman GFC filters pre-soaked in 0.3% (w/v) polyethyleneimine [14]. The filters were washed twice with 2 ml phosphate-buffered saline; filtration and washing was accomplished within 20 s. The filters were counted in 5 ml Optiphase 'safe' scintillant in a Packard scintillation spectrometer.

^{125}I-α-Bungarotoxin binding to P2 membrane fractions was measured using a centrifugation assay [15]. For the locust P2 membrane fraction, samples (0.25 ml) were incubated in exactly the same way as rat samples. However, the unbound radioligand was removed by filtration on GFC filters pre-soaked in polyethyleneimine (0.3%, w/v). The filters were measured for radioactivity in an LKB 1280 Ultrogamma counter.

^{125}I-α-Bungarotoxin binding to *Torpedo* nAChR was carried out as described [3].

3. RESULTS

3.1. *Saturable binding of (−)-[^{3}H]nicotine and ^{125}I-α-bungarotoxin*

Saturation binding experiments were performed on the P2 membrane fractions from rat brain and locust ganglia using (−)-[^{3}H]nicotine and ^{125}I-α-bungarotoxin. The binding data for both tissues are listed in table 1. The K_d for (−)-[^{3}H]nicotine binding to locust ganglion P2 membranes was higher than to membranes from rat brain. ^{125}I-α-Bungarotoxin bound to the locust tissue with high affinity comparable to that for binding to rat brain. In the case of locust membranes, the B_{max} values for both ligands were an order of magnitude more than those observed in rat brain P2 membranes (table 1). In both tissues, B_{max} for (−)-[^{3}H]nicotine binding was 2–4-times that for ^{125}I-α-bungarotoxin binding to the same membrane preparation.

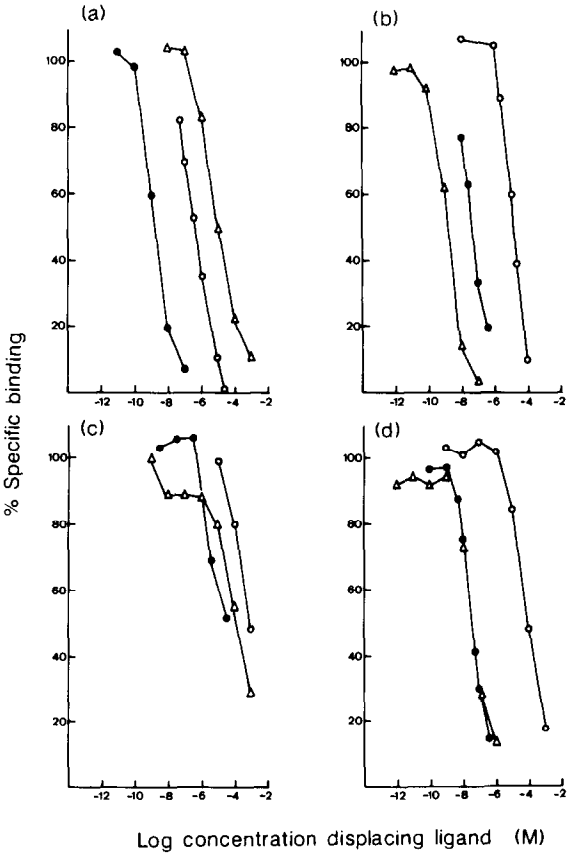

Fig.1. Competition binding assays with MLA and anatoxin-a. Competition assays for (a) (−)-[^{3}H]nicotine and (b) ^{125}I-α-bungarotoxin binding to rat brain P2 membranes, and (c) (−)-[^{3}H]nicotine and (d) ^{125}I-α-bungarotoxin binding to locust ganglion P2 membranes were carried out in the presence of MLA (△), (+)-anatoxin-a (●) and (−)-anatoxin-a (○). Results are taken from a representative experiment, with each ligand concentration assayed in triplicate.

3.2. *Inhibition of binding by MLA and (+)-anatoxin-a*

In competition assays, (+)-anatoxin-a was a potent competitor of (−)-[^{3}H]nicotine binding to rat brain P2 membranes (fig.1a), being at least an order of magnitude more potent than (−)-nicotine (table 2). The toxin was correspondingly more potent than (−)-nicotine in blocking ^{125}I-α-bungarotoxin binding to rat brain (fig.1b) but both agonists have micromolar rather than nanomolar affinities at this site (table 2). In locust ganglia, (+)-anatoxin-a displayed a 5–10-fold higher potency at the ^{125}I-α-bungarotoxin-binding site (fig.1d) compared to the corresponding binding site in rat brain (fig.1b), but the (−)-[^{3}H]nicotine-binding site in locust was largely insensitive to (+)-anatoxin-a (fig.1c). At each of these sites (−)-anatoxin-a had a much weaker affinity, and the activity observed with the (−) enantiomer can be ascribed to residual traces of (+)-anatoxin-a.

MLA had a high affinity for the locust neuronal ^{125}I-α-bungarotoxin-binding site (fig.1d) and was similarly potent in competing for the ^{125}I-α-bungarotoxin-binding site in rat brain (fig.1b). However, in competition assays for ^{125}I-α-bungarotoxin binding to purified *Torpedo* nAChR, MLA had a K_i of $1.15 \pm 0.65 \times 10^{-6}$ M ($n = 3$). MLA was a much weaker competitor of (−)-[^{3}H]nicotine binding to both rat brain (fig.1a) and locust ganglia (fig.1c).

3.3. *Pharmacological specificity of (−)-[^{3}H]-nicotine and ^{125}I-α-bungarotoxin sites*

A further comparison of the binding sites for

Table 2

Inhibition of (−)-[^{3}H]nicotine and ^{125}I-α-bungarotoxin binding to rat brain and locust ganglion P2 membranes by cholinergic drugs

Displacing ligand	K_i (M)			
	(−)-[^{3}H]Nicotine		^{125}I-α-Bungarotoxin	
	Rat brain	Locust ganglia	Rat brain	Locust ganglia
(+)-Anatoxin-a	3.4×10^{-10}	$>10^{-5}$	9.1×10^{-8}	1.6×10^{-8}
(−)-Anatoxin-a	3.9×10^{-7}	$>10^{-3}$	2.1×10^{-5}	1.0×10^{-4}
(−)-Nicotine	1.5×10^{-8}	7.7×10^{-7}	5.1×10^{-6}	5.1×10^{-7}
(+)-Nicotine		4.7×10^{-6}		1.5×10^{-5}
MLA	3.7×10^{-6}	2.5×10^{-4}	1.4×10^{-9}	1.8×10^{-8}
DMPP	4.0×10^{-7}	2.0×10^{-4}	2.3×10^{-6}	3.8×10^{-6}
Dihydro-β-erythroidine	1.3×10^{-6}	1.8×10^{-4}	2.5×10^{-5}	1.3×10^{-8}
Decamethonium	1.2×10^{-4}		2.0×10^{-4}	
Hexamethonium	3.9×10^{-3}		8.8×10^{-4}	
Pentolinium	3.3×10^{-4}		1.5×10^{-3}	
Pempidine	2.6×10^{-3}		2.5×10^{-3}	
Mecamylamine	1.0×10^{-2}		1.5×10^{-3}	
α-Bungarotoxin	$>10^{-6}$	$>10^{-6}$	1.0×10^{-9}	8.9×10^{-9}
Atropine		8.2×10^{-5}		
TEA		$>10^{-3}$		$>10^{-4}$

Competition binding assays were performed as described in section 2 using (−)-[^{3}H]nicotine at 10 nM (rat brain) or 50 nM (locust ganglia) and ^{125}I-α-bungarotoxin at 1 nM. IC_{50} values were derived from linear transformations of dose-response curves; K_i values were derived from IC_{50} values [16], assuming K_d values for [^{3}H]nicotine binding to P2 membranes of 8.0 nM (rat brain) or 130 nM (locust ganglia), and 1.0 nM for ^{125}I-α-bungarotoxin binding to both tissues. Data are the means of at least 3 independent determinations for rat brain membranes and 2 determinations for locust ganglia, carried out in triplicate

both (−)-[^{3}H]nicotine and ^{125}I-α-bungarotoxin was undertaken by determining a wider pharmacological profile (table 2). The α-bungarotoxin-binding site in both tissues displayed comparable drug sensitivities with moderate competition by nicotinic agonists, including nicotine, and poor inhibition by antagonists, with the exception of α-bungarotoxin itself and dihydro-β-erythroidine. The invertebrate site exhibited slightly higher sensitivities to most of the drugs tested when compared with the rat brain site. In contrast, (−)-[^{3}H]nicotine-binding sites in the two tissue preparations differed markedly; whereas binding to mammalian brain was generally very sensitive to nicotinic agonists, (−)-[^{3}H]nicotine binding to the invertebrate preparation was largely unaffected by all the drugs tested. Furthermore, [^{3}H]nicotine binding to rat brain membranes was characterized by considerable stereoselectivity for the (−) enantiomer of nicotine [2,3]. This site in locust ganglion membranes displayed little preference for (−)-nicotine (table 2) and had low sensitivity to nicotinic agonists. Indeed, in the locust the α-bungarotoxin-binding site showed greater stereoselectivity for (−)-nicotine than did the nicotine-binding site.

4. DISCUSSION

(+)-Anatoxin-a and MLA interact quite differently with the (−)-[^{3}H]nicotine-binding site and the ^{125}I-α-bungarotoxin-binding site in rat brain. At the neuromuscular junction, (+)-anatoxin-a is a potent nicotinic agonist and its activity is stereospecific, residing in the (+) enantiomer of the alkaloid [9,10]. We have recently reported [11] that (+)-anatoxin-a activates neurones in the hippocampus and brain stem. We show here that the high-affinity (−)-[^{3}H]nicotine-binding site in rat brain is particularly sensitive to (+)-anatoxin-a (fig.1a), whereas the corresponding site in locust ganglia is virtually insensitive (fig.1c). At the α-bungarotoxin site, (+)-anatoxin-a was found to be an effective inhibitor of binding in both locust ganglia and rat brain. A previous study on rat brain showed that racemic anatoxin-a had an even lower affinity for the muscarinic site labelled by [^{3}H]quinuclidinyl benzilate [17].

MLA has a high affinity ($K_i = 2.5 \times 10^{-10}$ M) for the α-bungarotoxin-binding site in housefly heads [7] and an ED_{50} value in the micromolar range at the mammalian neuromuscular junction [8]. We observed that in rat brain MLA is much more effective at the α-bungarotoxin-binding site (fig.1b) than at the nicotine site (fig.1a). This is the converse of the effects seen with (+)-anatoxin-a. In locust ganglia however, the α-bungarotoxin site was equally sensitive to MLA and anatoxin-a (fig.1d) while the nicotine site was insensitive to both toxins (fig.1c). From these results, it seems that MLA is a highly selective toxin for the α-bungarotoxin-binding site, a preference previously only demonstrated by the snake venom α-neurotoxins. Moreover, MLA also displays a much higher affinity for the neuronal α-bungarotoxin-binding site than for the nAChR in *Torpedo*, suggesting that it may be generally useful as a preferential probe for the α-toxin receptor in the CNS of both vertebrates and invertebrates. Our results for ^{125}I-α-bungarotoxin binding to locust ganglion P2 membranes are comparable to the published values for other insects [5] and are similar to those for rat brain P2 membranes. In vertebrates α-bungarotoxin is a potent and well-characterized nicotinic antagonist only at peripheral sites; the functional significance of neuronal α-bungarotoxin-binding sites is unknown. In contrast to mammalian brain, a blockade of excitatory synaptic transmission by α-bungarotoxin has been demonstrated electrophysiologically in the cockroach CNS [18]. However, other workers have reported central nicotinic responses in insects that are insensitive to α-bungarotoxin [19].

Unlike the α-bungarotoxin-binding site, the (−)-[^{3}H]nicotine-binding sites differ markedly in rat brain and locust ganglia. Thus, in rat brain the binding of (−)-[^{3}H]nicotine exhibited greater sensitivity to many of the nicotinic agonists than did the ^{125}I-α-bungarotoxin-binding site, further emphasizing the presence of two distinct sites in this tissue. This is in agreement with autoradiographic studies on rat brain slices using these two ligands which show that the distribution of (−)-[^{3}H]nicotine- and ^{125}I-α-bungarotoxin-binding sites is distinctly different [20,21] and with demonstrations that the two binding sites are associated with different proteins [3,22]. It has also been shown that the rat brain high-affinity

(−)-[^{3}H]nicotine-binding site has very similar characteristics to a [^{3}H]acetylcholine-binding site [2,23]. The behavior of the (−)-[^{3}H]nicotine-binding site in the locust is in marked contrast; thus (−)-[^{3}H]nicotine binds with lower affinity and the binding did not fully saturate over the range 0–100 nM. The locust site exhibited very low affinity for all the ligands used and only (−)- and (+)-nicotine were effective in displacing the bound (−)-[^{3}H]nicotine; even then the stereospecificity of their effectiveness was very slight compared to the rat brain site [3].

Whereas in the rat brain the nicotine-binding site is a strong candidate for a physiological nAChR (see [1]), in the locust this seems not to be the case. Nicotine certainly possesses insecticidal properties but our findings with MLA, which is a more potent insecticide [7] and a more potent inhibitor of ^{125}I-α-bungarotoxin binding, suggests that the target is the α-bungarotoxin-binding site rather than the (−)-[^{3}H]nicotine-binding site. Thus we would conclude that in the insect ganglia there is an acetylcholine receptor labelled by ^{125}I-α-bungarotoxin that shares pharmacological specificity with its mammalian counterparts in peripheral and central nervous systems. Whether such a receptor is, in evolutionary terms, the parent protein of the present-day vertebrate receptors [24] or whether both vertebrate and insect nAChRs have evolved from a single more ancient protein remains to be elucidated. The novel neurotoxins MLA and (+)-anatoxin-a are likely to be useful probes in the quest for a better understanding of the relationships between neuronal nicotinic receptors.

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