Conversion of the sodium channel activator aconitine into a potent α7-selective nicotinic ligand

David J. Hardick[a], Gary Cooper[a], Toby Scott-Ward[b], Ian S. Blagbrough[a], Barry V.L. Potter, Susan Wonnacott[b,*]

[a]*School of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK*
[b]*School of Biology and Biochemistry, 4 West, University of Bath, Bath BA2 7AY, UK*

Received 4 April 1995

Abstract **Methyllycaconitine (MLA) is a competitive antagonist of nicotinic acetylcholine receptors, with a remarkable preference for neuronal [^{125}I]αBgt binding sites. We have begun to investigate the structural basis of its potency and subtype selectivity. MLA is a substituted norditerpenoid alkaloid linked to a 2-(methylsuccinimido)benzoyl moiety. Hydrolysis of the ester bond in MLA to produce lycoctonine diminished affinity for rat brain [^{125}I]αBgt binding sites 2500-fold and abolished affinity for [^{3}H]nicotine and muscle [^{125}I]αBgt binding sites. The voltage-gated Na^+ channel activator aconitine, also a norditerpenoid alkaloid, but with significant structural differences from lycoctonine, displayed comparable weak or absent nicotinic activity. Addition of a 2-(methylsuccinimido)benzoyl sidechain to *O*-demethylated aconitine, to mimic MLA, abolished Na^+ channel activation and conferred nanomolar affinity for brain [^{125}I]αBgt binding sites, comparable to that of MLA. We propose that the ester-linked 2-(methylsuccinimido)benzoyl group is necessary for nicotinic potency, but α7 selectivity resides in the norditerpenoid core of the molecule.**

Key words: Methyllycaconitine; Aconitine; α-Bungarotoxin; [^{3}H]Nicotine; Nicotinic receptor subtype; Tetrodotoxin; Veratridine

1. Introduction

The norditerpenoid alkaloid methyllycaconitine (MLA; Fig. 1a), which occurs naturally in *Delphinium* sp., is a potent competitive antagonist of nicotinic acetylcholine receptors (nAChR) [1]. MLA is remarkable in being highly selective for neuronal nAChR subtypes that are sensitive to the snake toxin α-bungarotoxin (αBgt) in both vertebrates and insects [2,3,4]. The nanomolar affinity with which MLA binds to, and inhibits, this class of nAChR [5] makes it an important probe, and we are interested in understanding the structural basis of its potency and selectivity.

Aconitine (Fig. 1c) is another norditerpenoid alkaloid, found in *Aconitum* sp. [6]. Its principal target is considered to be voltage-gated Na^+ channels, with which it interacts in such a way as to prevent the inactivation of the channel, thus prolonging depolarisation. Therefore, its mechanism of action is akin to that of batrachotoxin and veratridine [7]. Despite their similar trivial names, there are several structural differences between aconitine and MLA. These two alkaloids differ in oxygenation pattern, stereochemistry, and the presence of bulky functional groups, such as benzoyl and acetyl, which are present in aconitine but not in MLA. Furthermore, the norditerpenoid portion of MLA is linked, via the C18 oxygen atom, to a 2-(methylsuccinimido)benzoyl moiety, whereas aconitine contains an *O*-methyl ether at C18 (Fig. 1c).

We have previously proposed [3] that a homocholine ester motif formed between the alkaloid nitrogen atom and the acyl group may confer nicotinic potency, by complying with the requirements of a proposed nicotinic pharmacophore [8]. One test of this hypothesis is to hydrolyse the ester, producing lycoctonine (Fig. 1b). This is a weak competitor at [^{125}I]αBgt binding sites in an insect preparation [9]. A recent evaluation of a series of norditerpenoid alkaloids isolated from *Delphinium* for their potencies in inhibiting [^{125}I]αBgt binding to rat brain and housefly head membranes [4] indicated that the most potent compounds retained the 2-(methylsuccinimido)benzoyl moiety at C18. The potent compounds in this study were, however, similar to MLA with respect to substituents in the norditerpenoid core of the molecule.

We have synthesised, for the first time, two anthranilate esters of aconitine (Fig. 1d,e), in order to mimic the structure of MLA. We report here that addition of the full 2-(methylsuccinimido)benzoyl sidechain abolishes the ability to activate voltage-gated Na^+ channels and produces a subtype-selective nicotinic ligand with potency comparable to that of MLA.

2. Materials and methods

2.1. Chemistry

MLA was hydrolysed to lycoctonine in 77% yield by base-catalysed hydrolysis (2 N KOH in ethanol, 25 h at 20°C; purified by silica gel chromatography using dichloromethane/methanol/NH_4OH 100:10:1 as eluant). The detailed chemistry for the *O*-demethylation and anthranoylation of aconitine, to give 3-deoxy-18-*O*-desmethyl(2-amino-benzoyl)aconitine (Fig. 1d) and 3-deoxy-18-*O*-desmethyl[2-(methylsuccinimido)benzoyl]-aconitine (Fig. 1e), has been reported elsewhere [10,11].

2.2. Nicotinic binding assays

Competitive binding assays were carried out as previously described for neuronal nAChR labelled with [^{125}I]αBgt or [^{3}H]nicotine in rat brain membranes [2] and for muscle nAChR labelled with [^{125}I]αBgt in rat muscle extract [12]. IC_{50} values were determined by curve fitting the Hill equation to the data; K_i values were derived from IC_{50} values according to the Cheng and Prusoff equation [13].

2.3. Indirect assays of Na^+ channel activation

The tetrodotoxin-sensitive stimulation of [^{3}H]dopamine release from brain slices by Na^+ channel activators was employed to evaluate the interaction of norditerpenoid alkaloids with this voltage-sensitive ion channel. Tissue slices (0.2 mm) were prepared from rat frontal cortex and loaded with [^{3}H]dopamine (0.1 μM; 45 Ci/mmol) in Krebs–Ringer containing 80 μM pargyline and 1.1 mM ascorbic acid, for 25 min at 37°C. Aliquots (200 μl; ~15 mg tissue) were incubated with drug, in the presence and absence of tetrodotoxin (TTX; 10 μM), for 15 min at

*Corresponding author. Fax: (44) (1225) 82-6449.
E-mail: s.wonna-cott@bath.ac.uk

SSDI 0014-5793(95)00426-2

37°C, followed by dilution with cold buffer and brief centrifugation. Radioactivity released into the medium above basal release was taken as an index of Na^+ channel activation. Release was calculated as % of total radioactivity in the tissue at time zero.

2.4. Materials

αBgt, veratridine and TTX were purchased from the Sigma Chemical Co. (Poole, Dorset, UK). αBgt was iodinated to a specific activity of 700 μCi/mmol. [^{3}H]Dopamine was purchased from Amersham Int. (Amersham, Bucks., UK). Aconitine was purchased from Aldrich Chemical Co. MLA was purified from a Garden Hybrid *Delphinium* strain as previously described [14]. Potential ligands were dissolved in ethanol to a stock concentration of 10^{-3}M and stored at 4°C.

3. Results

Lycoctonine was prepared by base-catalysed hydrolysis of MLA. In order to facilitate the esterification of aconitine, it was first necessary to deoxygenate at C3 and *O*-demethylate at C18 to give 3-deoxy-18-*O*-desmethylaconitine [10]. Subsequently, anthranoyl and 2-(methylsuccinimido)benzoyl groups were added [11]: the products are 3-deoxyaconitine based analogues of MLA. The 2-(methylsuccinimido)benzoyl aconitine (Fig. 1e) was homogeneous by thin layer chromatography (TLC), and gave satisfactory mass, ^{13}C and ^{1}H NMR spectra. The anthranoyl aconitine (Fig. 1d) gave a satisfactory ^{1}H NMR spectrum, but contained trace impurities as judged by TLC (SiO_2, 10% methanol/dichloromethane). Importantly, *Aconitum* sp. which are the source of aconitine, do not contain MLA, ruling it out as a potential contaminant.

These semi-synthetic products, together with the parent compounds aconitine and MLA, were assayed for nicotinic potency in competition binding assays for both muscle and neuronal nAChR. [^{125}I]αBgt and [^{3}H]nicotine binding define the two major subtypes of nAChR found in mammalian brain. The inhibition curves are depicted in Fig. 2 and K_i values are given in Table 1. MLA, isolated and purified in this laboratory as the free base [14], inhibited [^{125}I]αBgt binding to rat brain membranes with a K_i of 4 nM, and was at least 3 orders of magnitude less potent at [^{3}H]nicotine binding sites or muscle nAChR. These data are in good agreement with our previous evaluations of MLA [2,3]. In contrast, aconitine (Fig. 1c) was only a weak competitor at neuronal [^{125}I]αBgt binding sites (K_i = 19 μM) and had no appreciable activity at any of the other nAChR subtypes examined. The norditerpenoid alkaloid lycoctonine (Fig. 1b) showed a similar binding profile (K_i = 10 μM at [^{125}I]αBgt binding sites) and was a feeble competitor compared with MLA.

Addition of 2-aminobenzoyl and 2-(methylsuccinimido)benzoyl groups to 3-deoxy-18-*O*-desmethylaconitine progressively enhanced nicotinic potency at brain [^{125}I]αBgt sites, with K_i values of 0.3 μM and 6 nM, respectively, compared with 4 nM for MLA. At neuronal [^{3}H]nicotine and muscle [^{125}I]αBgt binding sites only the 2-(methylsuccinimido)benzoyl-analogue displayed any potency, inhibiting [^{125}I]αBgt binding to muscle with a K_i of 42 μM.

In an indirect assay of Na^+ channel activation, based on the TTX-sensitive stimulation of [^{3}H]dopamine release from frontal cortex slices, aconitine produced a concentration-dependent release of radiolabel, with an EC_{50} of 21.2 ± 1.2 μM (Fig. 3a).

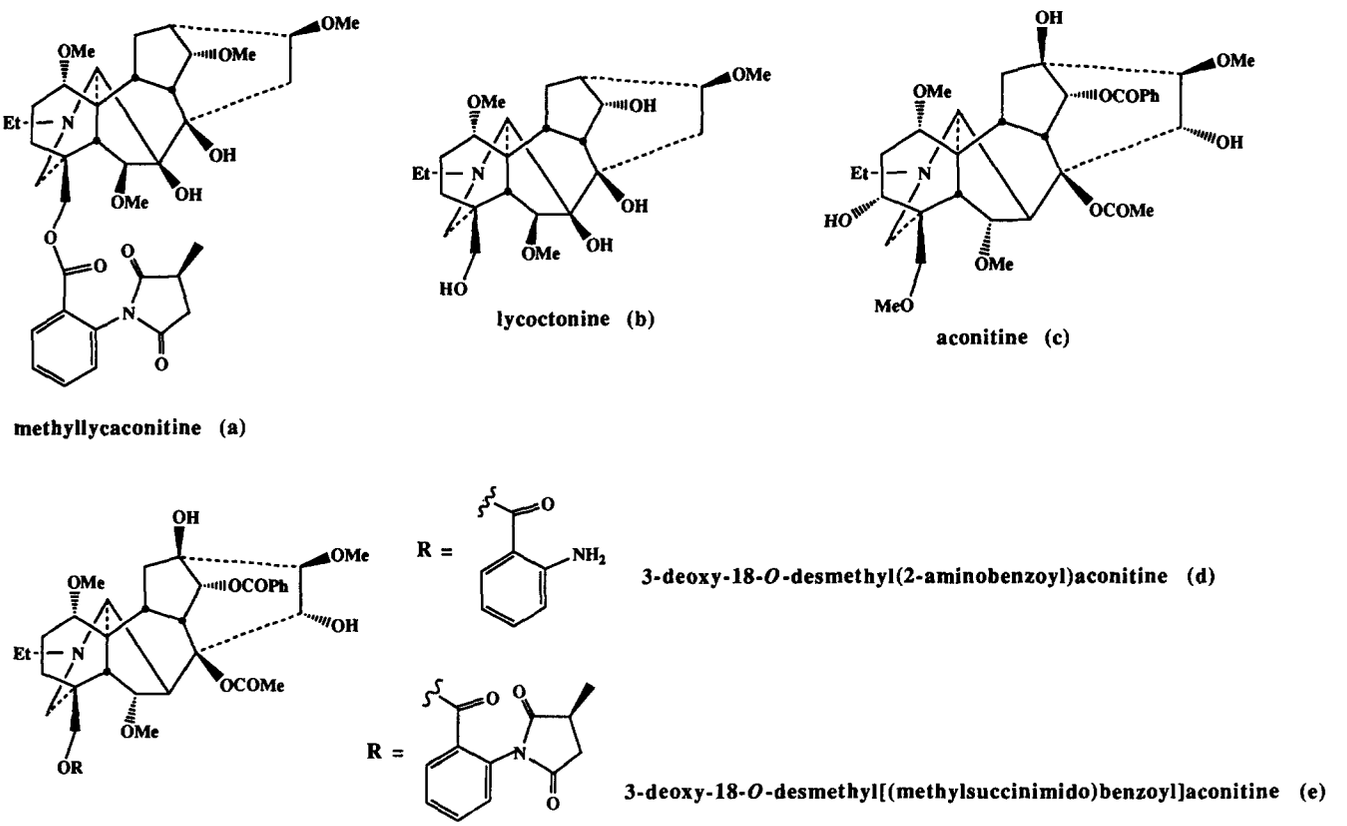

Fig. 1. Chemical structures of (a) MLA, (b) lycoctonine, (c) aconitine, (d) 3-deoxy-18-*O*-desmethyl(2-aminobenzoyl)aconitine; (e) 3-deoxy-18-*O*-desmethyl[2-(methyl-succinimido)benzoyl]aconitin e.

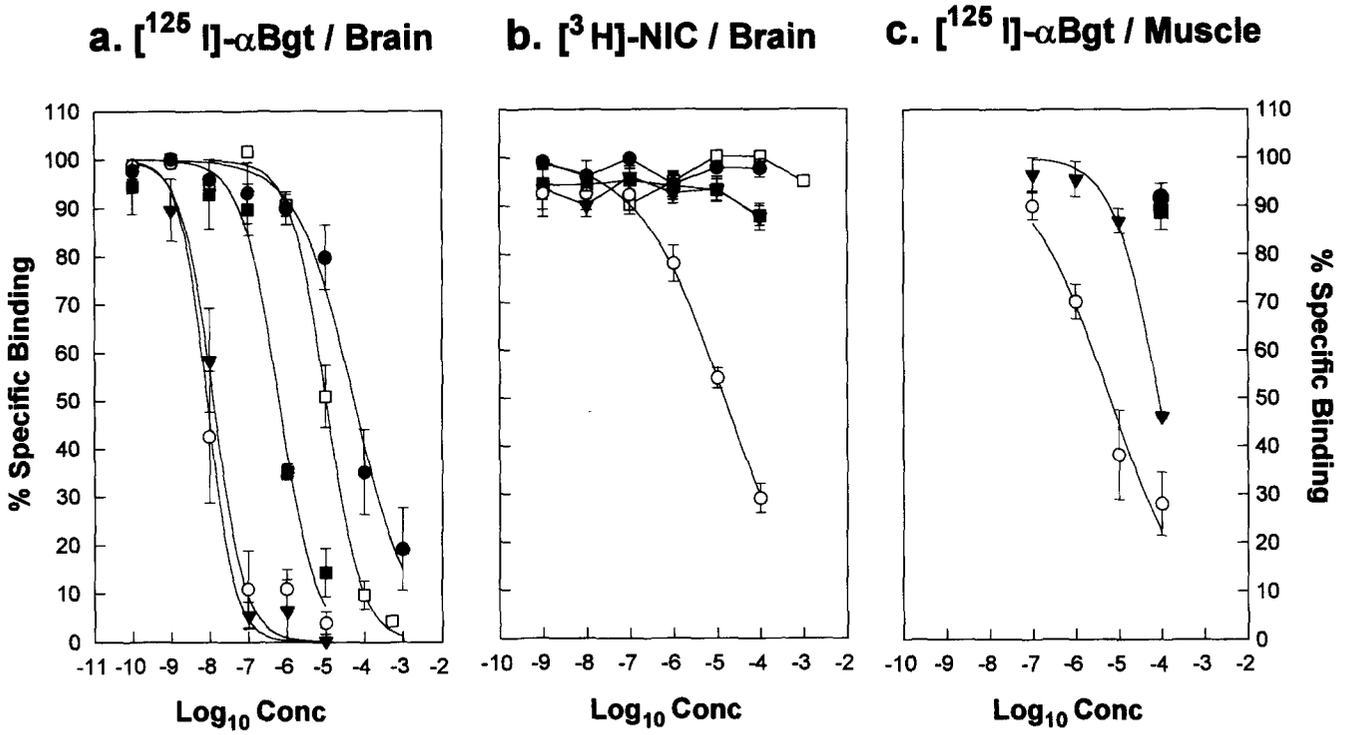

Fig. 2. Competition binding assays for (a) [^{125}I]αBgt binding sites in rat brain membranes; (b) [^{3}H]nicotine binding sites in rat brain membranes; (c) [^{125}I]αBgt binding sites in rat muscle extract. Binding assays were carried out as described in section 2. Each point is the mean of 3 independent assays, vertical bars indicate the S.E.M. (○) MLA; (□) lycoctonine; (●) aconitine; (■) 3-deoxy-18-*O*-desmethyl(2-aminobenzoyl)aconitine; (▼) 3-deoxy-18-*O*-desmethyl[2-(methylsuccinimido)-benzoyl]aconitin e.

It was less efficacious and less potent than veratridine, which gave an EC_{50} of 3.1 ± 0.3 μM. The effects of both drugs were abolished by coincubation with TTX. In contrast, MLA, lycoctonine and 2-(methylsuccinimido)benzoyl aconitine had no effect on [^{3}H]dopamine release (Fig. 3b).

4. Discussion

MLA is a potent nicotinic ligand with selectivity for neuronal αBgt-sensitive nAChR. Hydrolysis of the C18 ester bond to produce lycoctonine resulted in a 2500-fold reduction in potency. This agrees with the differential potencies of MLA and lycoctonine in inhibiting [^{125}I]αBgt binding to fly head homogenate, although this insect nAChR may be even more sensitive to MLA than its counterpart in rat brain [9]. Lycoctonine was without activity at brain [^{3}H]nicotine binding sites, and has been reported to be devoid of neuromuscular blocking activity in the rat phrenic nerve–diaphragm preparation [15]. Lycoctonine did not evoke the release of [^{3}H]dopamine, suggesting that the structural differences between this alkaloid and aconitine were sufficient to render lycoctonine incapable of activating voltage-gated Na^+ channels.

Aconitine was comparable to lycoctonine in its weak nicotinic activity. Aconitine inhibits [^{125}I]αBgt binding to fly head homogenate with a K_i of 270 μM [9], although it has been reported to be more potent in depressing a rat neuromuscular response [15]. Given its actions at voltage-gated Na^+ channels, it is not clear that this neuromuscular depression reflects an interaction with nAChR [16]. However, the generation of nanomolar affinity for rat brain [^{125}I]αBgt binding sites, comparable to that of MLA, by addition of a 2-(methylsuccinimido)benzoyl moiety is a striking indication that this esterification is responsible for nicotinic potency. This result is the first demonstration that significant changes can be made to the norditerpenoid core of the ligand without loss of activity, providing the 2-(methylsuccinimido)benzoyl moiety is present. The anthranoyl intermediate (lacking the methylsuccinyl ring) had lower activity, and we propose, therefore, that the ester bond alone is insuffi-

Table 1
Binding affinities of MLA, aconitine and related structures at nAChR

	Brain [^{125}I]αBgt	Brain [^{3}H]nicotine K_i (M)*	Muscle [^{125}I]αBgt
MLA	$4.3 \pm 1.5 \times 10^{-9}$	$7.5 \pm 1.5 \times 10^{-6}$	$3.0 \pm 1.1 \times 10^{-6}$
Lycoctonine	$1.0 \pm 0.1 \times 10^{-5}$	$> 1 \times 10^{-4}$	n.d.
Aconitine	$1.9 \pm 0.4 \times 10^{-5}$	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$
3-Deoxy-18-*O*-desmethyl(2-aminobenzoyl)aconitine	$3.1 \pm 0.7 \times 10^{-7}$	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$
3-Deoxy-18-*O*-desmethyl[2-(methylsuccinimido)-benzoyl]aconitine	$6.0 \pm 1.5 \times 10^{-9}$	$> 1 \times 10^{-4}$	$4.2 \pm 0.5 \times 10^{-5}$

*Mean ± S.E.M. from 3 independent assays.
n.d. = not determined.

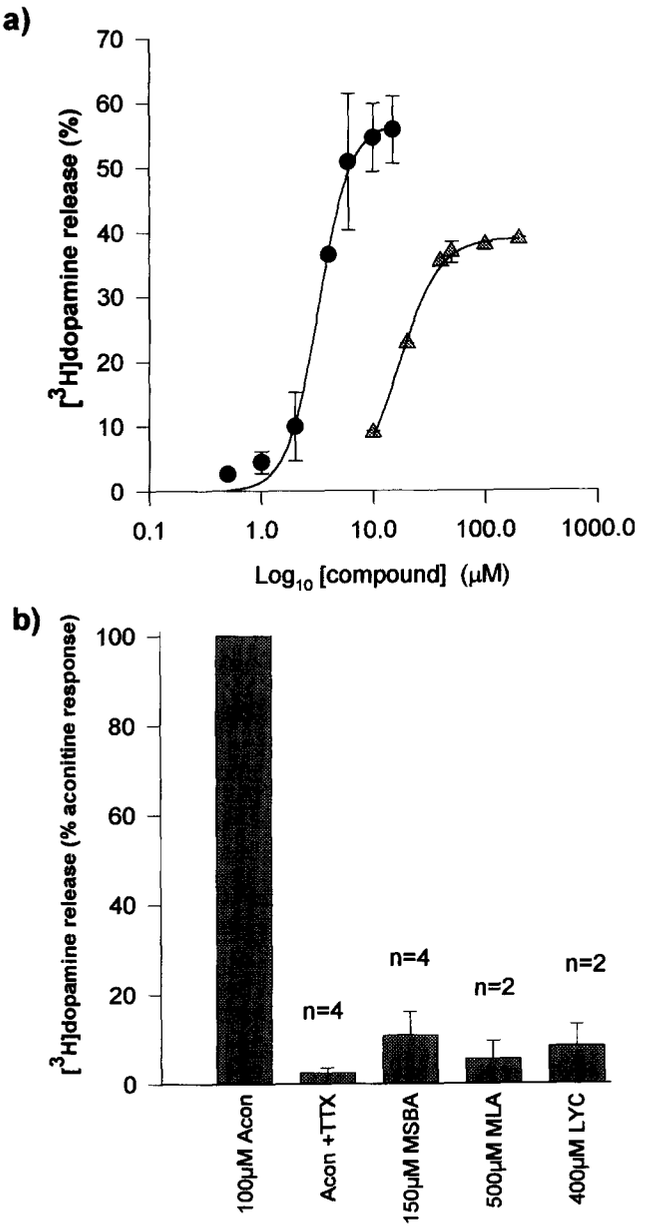

Fig. 3. Stimulation of [^{3}H]dopamine release from rat cortical slices by activation of TTX-sensitive Na^+ channels. (a) Concentration dependence of veratridine (●) and aconitine (▲) stimulated [^{3}H]dopamine release. Cortical slices were loaded with [^{3}H]dopamine and incubated with increasing concentrations of drug for 15 min. [^{3}H]Dopamine released into the medium was measured and corrected for basal release, determined in the presence of TTX. Values are the mean ± range from 2 independent assays. (b) Comparison of norditerpenoid alkaloids. MLA (500 μM), lycoctonine (LYC; 400 μM) and 3-deoxy-18-*O*-des-methyl[2-(methylsuccinimido)benzoyl]aconitine (MSBA; 150 μM) were compared with a maximally effective concentration of aconitine (Acon; 100 μM) for their ability to evoke [^{3}H]dopamine release from cortical slices. TTX sensitivity was demonstrated by coincubation of aconitine (100 μM) with 10 μM TTX (Acon+TTX). Values are presented as a % of the response to aconitine, determined in parallel, and are the mean ± range or S.E.M. of the number of independent assays indicated.

cient for full potency. This is in accord with the preference shown for the succinimide substituent in the series of norditerpenoid alkaloids similar to MLA, published recently by Kukel and Jennings [4].

Comparison of activities at the three nAChR sites assayed in the present study shows that the preference for neuronal [^{125}I]αBgt binding sites is conserved across the series of compounds examined. This preference is evident even in aconitine and lycoctonine, which share a common norditerpenoid carbon core, although they differ in several functional groups. From the binding data, we propose that the rigid norditerpenoid ring structure is the most important determinant of α7 selectivity. However, the substituents probably influence activity at [^{3}H]nicotine binding sites, as the 2-(methylsuccinimido)benzoyl derivative of aconitine shows more subtype-selectivity than MLA. Moreover, such differences must explain the failure of lycoctonine to activate TTX-sensitive Na^+ channels, in contrast to aconitine. The addition of the 2-(methylsuccinimido)benzoyl group to aconitine abolished its ability to stimulate TTX-sensitive [^{3}H]dopamine release, demonstrating that the pharmacophores for the two sites (voltage-gated Na^+ channel and nAChR) have little overlap.

This study defines the norditerpenoid core of MLA as the major determinant of α7 nAChR selectivity, and establishes the prime importance of the 2-(methylsuccinimido)benzoyl substituent for nicotinic potency. The conversion of aconitine to a potent nicotinic ligand, comparable to MLA, with concomitant loss of TTX-sensitive Na^+ channel activation, by addition of a 2-(methylsuccinimido)benzoyl sidechain is a major step towards the rational design of nicotinic subtype-selective drugs.

Acknowledgements: We thank the Wellcome Trust (Project Grant 036214) and EC for financial support. B.V.L.P. is a Lister Institute Research Professor.

References

[1] Wonnacott, S., Albuquerque, E.X. and Bertrand, D. (1993) in: Methods in Neurosciences, vol 12 (Conn, M. ed.) pp. 263–275, Academic Press, New York.
[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] Ward, J.M., Cockcroft, V.B., Lunt, G.G., Smillie, F.S. and Wonnacott, S. (1990) FEBS Lett. 270, 45–48.
[4] Kukel, C.F and Jennings, K.R. (1994) Can. J. Physiol. Pharmacol. 72, 104–107.
[5] Alkondon, M., Pereira, E.F.R., Wonnacott, S. and Albuquerque, E.X. (1992) Mol. Pharmacol. 41, 802–808.
[6] Benn, M.H. and Jacyno, J.M. (1983) in: Alkaloids: Chemical and Biological Perspectives, vol. 1 (Pelletier, S.W. ed.) Wiley, New York.
[7] Catterall, W.A. (1980) Annu. Rev. Pharmacol. Toxicol. 20, 15–43.
[8] Sheridan, R.P., Nilakantan, R., Dixon, J.S. and Venkataraghavan, R. (1986) J. Med. Chem. 29, 899–906.
[9] Jennings, K.R., Brown, D.G. and Wright, D.P. (1986) Experientia 42, 611–613.
[10] Blagbrough, I.S., Hardick, D.J., Wonnacott, S. and Potter, B.V.L. (1994) Lett. 35, 3367–3370.
[11] Hardick, D.J., Blagbrough, I.S., Wonnacott, S. and Potter, B.V.L. (1994) Tetrahedron Lett. 35, 3371–3374.
[12] Garcha, H.S., Thomas, P., Spivak, C.E., Wonnacott, S. and Stolerman, I.P (1993) Psychopharmacology 110, 347–354.
[13] Cheng, Y.-C. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099–3108.
[14] Coates, P.A., Blagbrough, I.S., Hardick, D.J., Rowan, M.G., Wonnacott, S. and Potter, B.V.L. (1994) Tetrahedron Lett. 35, 8701–8704.
[15] Nambi Aiyar, V., Benn, M.H., Hanna, T., Jacyno, J., Roth, S.H. and Wilkens, J.L. (1979) Experientia 35, 1367–1368.
[16] Muroi, M., Kimura, I. and Kimura, M. (1990) Neuropharmacology 29, 567–572.