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HOMOANATOXIN: A POTENT ANALOGUE OF ANATOXIN-A

S. WONNACOTT,*† K. L. SWANSON,‡ E. X. ALBUQUERQUE,‡ N. J. S. HUBY,§ P. THOMPSON§ and T. GALLAGHER§

Departments of *Biochemistry and §Chemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K.; and ‡Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A.

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Abstract—The natural toxin anatoxin-a (AnTx) is a potent nicotinic agonist that is valuable for the study of nicotinic receptors. We have synthesized 2-(propan-1-oxo-1-yl)-9-azabicyclo[4.2.1]non-2-ene, the homologue of AnTx in which the side-chain is extended by one methylene unit from a methyl to an ethyl ketone. This chemistry would allow the generation of a tritiated product and the homologue, designated homoanatoxin (HomoAnTx), has been characterized here with that aim in mind. In competition binding assays at neuronal nicotinic ligand binding sites characterized by [^{3}H]nicotine and [^{125}I]-αbungarotoxin, HomoAnTx retained the same potency as the parent molecule, with K_i values of 7.5 nM and 1.1 μM, respectively. In contrast, it showed little inhibition of muscarinic binding defined by [^{3}H]-quinuclidinyl benzilate. HomoAnTx is a potent nicotinic agonist in frog muscle contracture assays, having four times the potency of carbamylcholine and one tenth of the activity of AnTx itself. The N-methylated version of HomoAnTx was more than two orders of magnitude weaker in both functional and binding assays. The successful synthesis of HomoAnTx with retention of high nicotinic potency offers a route for the generation of novel, potent radiolabelled nicotinic ligands.

(+)Anatoxin-a (AnTx‖) occurs naturally in toxic algal blooms of the freshwater cyanobacterium *Anabaena flos aquae*, and has been successfully synthesized *de novo* [1–4]. AnTx is a potent, stereoselective agonist of the nAChR at muscle endplates [5, 6], and is proving to be a valuable probe in the characterization of nAChR subtypes present in the CNS [7–10]. The semi-rigid structure of this bicyclic alkaloid makes it particularly attractive for structure–activity studies aimed at defining the agonist recognition site of nAChR. An extensive series of analogues with modifications to the acetyl side-chain, or N-methylation, has been generated by Rapoport and colleagues [11, 12]. None of these analogues was as potent as the parent compound (+)AnTx in functional tests on muscle [13–16] or in ligand binding assays of muscle and brain nAChR [16, 17]. In particular, these studies demonstrated the importance of the carbonyl function for receptor recognition and the potential of the extended side-chain to discriminate between peripheral and central subtypes of nicotinic receptors. However, this series did not include the simple homologue of AnTx, 2-(propan-1-oxo-1-yl)-9-azabicyclo[4.2.1]non-2-ene, designated homoanatoxin (HomoAnTx), whereby the chain length of the side-chain is extended by one methylene unit, from a methyl to an ethyl ketone (Fig. 1). Our interest in this modification is that it offers a straightforward means of incorporating a radiolabel into the AnTx skeleton, while retaining those structural features that are necessary for biological activity. The possibility of introducing tritium into the methyl group (C-11, Fig. 1) of HomoAnTx has the advantage that this is a chemically stable site and, since this site is not adjacent to the ketone function, loss of radioactivity by enolization is avoided. Tritiated HomoAnTx would be a useful addition to the limited complement of high affinity tritiated nicotinic agonists that are currently available [18, 19]. Radiolabelled nicotinic agonists, with the possible exception of [^{3}H]cytisine, have well-documented problems of stability and handling [18, 20, 21], whereas the stability of AnTx and the chemical considerations outlined above would commend [^{3}H]HomoAnTx in this respect.

Here we describe the synthesis of HomoAnTx, using the more readily accessible racemic material, and show that it is a potent agonist in muscle contracture assays; in ligand binding assays of neuronal nAChR HomoAnTx retains the high potency exhibited by AnTx itself.

MATERIALS AND METHODS

Chemistry

9-(Tert-butoxycarbonyl)-2-(propan-1-oxo-1-yl)-9-azabicyclo[4.2.1]non-2-ene (N-BOC HomoAnTx). To a solution of (±)-*N*-BOC AnTx (41 mg, 0.155 mmol) in THF (3 mL) was added LDA (0.19 mmol) in THF (0.8 mL) at 0° under nitrogen. After 1 hr, iodomethane (26 mg, 0.183 mmol) in THF (0.3 mL) was added and the mixture stirred at 0° for 2.5 hr. Saturated aqueous ammonium chloride (1 mL) was added, the mixture was poured into water (10 mL) and extracted with methylene chloride

† Corresponding author. Tel. (0225) 826391; FAX (0225) 826449.

‖ Abbreviations: AnTx, anatoxin-a; HomoAnTx, homoanatoxin; nAChR, nicotinic acetylcholine receptor; QNB, quinuclidinyl benzilate; carbachol, carbamylcholine; THF, tetrahydrofuran; LDA, lithium diisopropylamide.

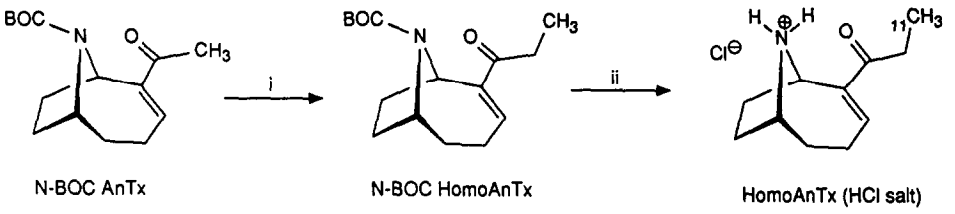

Fig. 1. Synthesis of HomoAnTx from its precursor, *N*-BOC AnTx. Reagents and conditions: i, LDA, THF, −78°, then CH_3I; ii, CF_3CO_2H, 0°, 15 min, then HCl.

(5 × 5 mL). The combined organic layers were dried (Na_2SO_4) and concentrated *in vacuo*. Chromatography gave reclaimed *N*-BOC AnTx (8 mg, 20%) and *N*-BOC HomoAnTx as a clear colourless oil (14 mg, 32%); R_f 0.60 (diethyl ether–petroleum). ν_{max} (thin film) 2980, 2940, 1670, 1395 cm^{-1}; δ_H (270 MHz, $CDCl_3$) (peaks split due to carbamate resonance) 1.03–1.16 (3H, m, $COCH_2CH_3$), 1.37 (6.3H, s, 0.7 $C(CH_3)_3$), 1.44 (2.7H, s, 0.3 $C(CH_3)_3$), 1.54–2.51 (8H, m, 4 × CH_2), 2.52–2.79 (2H, m, $COCH_2CH_3$), 4.22–4.49 (1H, m, C6*H*), 5.06–5.24 (1H, m, C1*H*) and 6.74–6.88 (1H, m, C3*H*); *m/z* (E.I.) 279 (M^+, 4%).

2-*(Propan*-1-*oxo*-1-*yl)* -9- *azabicyclo*[4.2.1]*non*-2-*ene hydrochloride (HomoAnTx)*. A solution of *N*-BOC HomoAnTx (13 mg, 47 μmol) in trifluoroacetic acid (1 mL) was stirred at 0° for 15 min under nitrogen. The excess of trifluoroacetic acid was removed *in vacuo*, the residue taken up in diethyl ether and saturated with dry HCl. The solvent was removed *in vacuo*, fresh diethyl ether added and the solution was again evaporated *in vacuo*. This process was repeated twice. Chromatography gave the HomoAnTx as the hydrochloride salt as a colourless glass (6 mg, 60%); R_f 0.15 (diethyl ether–methanol) (Found M^+, 179.1301. $C_{11}H_{17}NO$ requires M, 179.1309); ν_{max} (thin film) 3730–3150, 2920, 1665, 1590 cm^{-1}; δ_H (270 MHz, $CDCl_3$) 1.10 (3H, t, J 7.2 Hz, $COCH_2CH_3$), 1.20–2.88 (10H, m, 4 × CH_2 and $COCH_2CH_3$), 4.26–4.43 (1H, m, C6*H*), 5.23 (1H, br d, J 8.8 Hz, C1*H*) and 7.09–7.19 (1H, m, C3*H*); *m/z* (E.I.) 179 (M^+, 34%), 150 (M^+-C_2H_5, 39) and 57 (100).

N-*Methyl-HomoAnTx*. *N*-Methyl-HomoAnTx was prepared by a modifiction of the method used to prepare other N-methylated extended side-chain derivatives [4, 22].

Radioligand binding assays

Competition binding assays were carried out as previously described [23]. Briefly, P_2 membranes were prepared from frozen rat brains by differential centrifugation. The membranes were washed twice in 50 mM phosphate buffer, pH 7.4, containing protease inhibitors, and stored in aliquots at −20°.

For [^{3}H](−)nicotine binding assays, the tissue was diluted five-fold in 20 mM Hepes buffer, pH 7.5, containing NaCl (118 mM), KCl (4.8 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.2 mM), Tris (200 mM) [20]. Samples (0.25 mL; 0.5 mg protein) were incubated with serial dilutions of test drug, or with 10^{-3} M (−)nicotine to determine non-specific binding, for 10 min before addition of radioligand (final concentration 10 nM). The samples were incubated at 20° for 60 min, chilled and rapidly filtered using a Brandell cell harvester as described by Lippiello and Fernandes [24].

[^{125}I]αBungarotoxin binding was assayed using a centrifugation method [9, 25]. The P_2 membrane preparation was diluted 10-fold in 50 mM phosphate buffer, pH 7.4, and samples (0.5 mL; 0.5 mg protein) were preincubated with test drug or 10^{-6} M unlabelled αbungarotoxin prior to addition of [^{125}I]-αbungarotoxin (final concentration 1 nM). Following an incubation period of 2 hr at 20°, the samples were diluted with 0.5 mL buffer, centrifuged (2 min at 10,000 *g*; MSE Microcentaur bench centrifuge) and the pellet washed once with phosphate buffered saline (10 mM phosphate, pH 7.4, containing 140 mM NaCl).

[^{3}H]QNB binding was assayed by filtration. The tissue was diluted 200-fold in phosphate buffered saline, and aliquots (1 mL; 50 μg protein) were incubated with serial dilutions of HomoAnTx, or with 10^{-5} M atropine to determine non-specific binding, for 10 min before addition of radioligand (final concentration 0.2 nM). The samples were incubated at 37° for 2 hr, chilled and filtered as described above.

Linear transformation of competition curves was used to determine IC_{50} values which were converted to K_i values by the method of Cheng and Prusoff [26], assuming K_d values of 10 and 1 nM for [^{3}H]-(−)nicotine and [^{125}I]αbungarotoxin, respectively; these values approximate to K_d values previously determined in the same laboratory [9].

Muscle assays

Contracture and evoked twitch studies were performed *in vitro* using the *rectus abdominus* muscle from *Rana pipiens* with standard techniques and statistical analysis as described previously [6, 16]. Frog Ringer's solution was composed of (in mM): NaCl 115; KCl 2; $CaCl_2$ 1.8; NaH_3PO_4 1.3 and Na_2HPO_4 0.7, pH 7.4. The nicotinic potency of HomoAnTx was compared with that of carbamylcholine (carbachol).

Materials

(−)[*N-methyl*-^{3}H]Nicotine (78 Ci/mmol), 1-quinuclidinyl [*phenyl*-4-^{3}H]benzilate (42 Ci/mmol) and [^{125}I]Na were purchased from Amersham

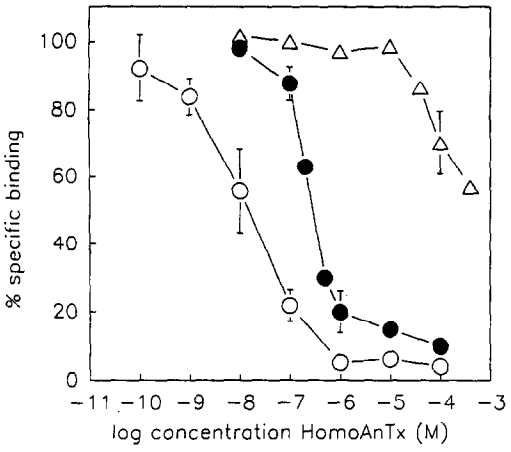

Fig. 2. Competition binding curves for HomoAnTx against [^{3}H]nicotine (○), [^{125}I]αbungarotoxin (●) and [^{3}H]QNB binding (△) to rat brain membranes. Serial dilutions of the analogue were assayed as described in Materials and Methods. Values are the means of three independent assays, with SE indicated by the vertical bars.

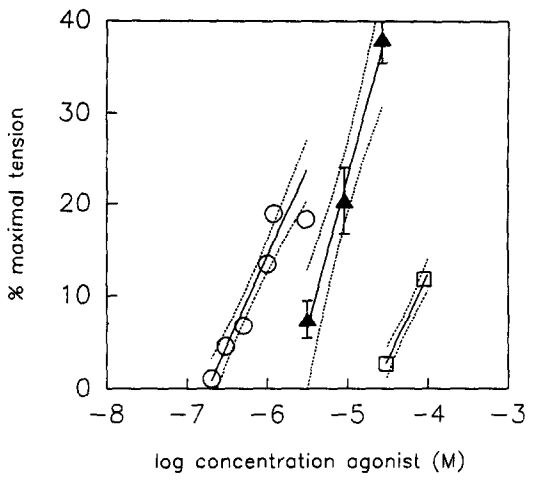

Fig. 3. Contracture potency of HomoAnTx and *N*-methyl-HomoAnTx in frog muscle preparations. Dilutions of HomoAnTx (○) and *N*-methyl-HomoAnTx (□) were compared with carbachol (▲) as previously described [16]. The least squares line (solid line) and 95% confidence interval (dotted curves) were calculated for each compound from the individual normalized data. The correlation coefficients for HomoAnTx, carbachol and *N*-methyl-HomoAnTx were 0.94, 0.88 and 0.95, respectively. The symbols represent the mean contracture tension ± SE for 4–6 muscles for each concentration tested, except for three additional points for HomoAnTx derived from single tests. Each drug was tested on muscles from three different frogs. Results from lower concentrations that failed to elicit contracture are not shown for clarity, and were not included in the statistical analysis.

International (Amersham, U.K.). αBungarotoxin was obtained from the Sigma Chemical Co. (Poole, U.K.) and was iodinated to a specific activity of 700 Ci/mmol.

RESULTS AND DISCUSSION

(±)HomoAnTx was prepared by deprotonation of racemic *N*-BOC AnTx, using LDA in THF and alkylation of the resulting enolate using iodomethane (Fig. 1) [4]. Removal of the BOC protecting group was accomplished using trifluoroacetic acid, and HomoAnTx was isolated as the corresponding hydrochloride salt. Since AnTx and HomoAnTx could not be readily separated, care was taken that N-BOC HomoAnTx was not contaminated with *N*-BOC AnTx prior to the deprotection step. As a result, both *N*-BOC HomoAnTx and HomoAnTx prepared in this way were homogenous as judged by highfield ^{1}H NMR.

Competition binding assays for neuronal nAChR sites labelled by [^{3}H]nicotine and [^{125}I]αbungarotoxin (Fig. 2) show that HomoAnTx is a potent nicotinic ligand. HomoAnTx is most potent at the [^{3}H]-nicotine site. The K_i value (7.5×10^{-9} M; Table 1) is comparable to the K_i for the parent compound (+)AnTx at the same site (3.5×10^{-9} M; [17]). Given that the homologue is racemic, whereas activity appears to reside in the (+) stereoisomer of AnTx [27], the two-fold difference in binding affinities of (+)AnTx and (±)HomoAnTx is compatible with the full retention of potency. Similarly, the K_i value of 2.6×10^{-7} M for HomoAnTx at the [^{125}I]αbungarotoxin binding site (Table 1) is comparable to the potency of the parent compound at this brain binding site (3.8×10^{-7} M; [17]). HomoAnTx has 35 times higher affinity at the [^{3}H]nicotine site than at the [^{125}I]αbungarotoxin site, and this is typical of the potency ratios of other AnTx analogues [17]. The high affinity [^{3}H]nicotine binding site is considered to represent the major subclass of functional nAChR in the brain [18, 23], whereas the [^{125}I]αbungarotoxin site has not yet been correlated with nicotinic functions in the CNS in the majority of instances [18].

The nicotinic selectivity of HomoAnTx is confirmed by the lack of inhibition of binding of the muscarinic ligand [^{3}H]QNB to brain membranes (Fig. 2): only 30% inhibition was observed at 10^{-4} M HomoAnTx. AnTx itself produces only weak and non-specific inhibition of muscarinic binding [28].

To assess the functional potency of HomoAnTx as a nicotinic agonist, its ability to elicit muscle contracture in the frog *rectus abdominus* preparation was determined. HomoAnTx was a potent agonist in this preparation (Fig. 3). Its relative potency to carbachol was 4.0 (95% confidence, range 2.0–7.5). Comparison with previous results, which showed (+)AnTx itself to have 110 (85–143) times the potency of carbachol [16], indicates that racemic HomoAnTx has only 1/25th of the potency of the parent compound. By analogy with (−)AnTx, it is probable that the (−) isomer of HomoAnTx is not only a very weak or impotent agonist but also possesses channel blocking activity [15]. Therefore, an allosteric antagonist action of (−)HomoAnTx may reduce the potency of the racemic mixture more than the two-fold that would be predicted for the

Table 1. Comparison of HomoAnTx and *N*-methyl-HomoAnTx

Drug	Brain binding sites K_i (M) [^{3}H]Nicotine	Brain binding sites K_i (M) [^{125}I]αBgt	Muscle contracture assays EC_{15} (M)
HomoAnTx	$7.5 \pm 3.5 \times 10^{-9}$	$2.6 \pm 1.2 \times 10^{-7}$	$1.15 \pm 0.12 \times 10^{-6}$
N-Methyl-HomoAnTx	$3.5 \pm 1.4 \times 10^{-6}$	ND	$>10^{-4}$

Values are the mean ± SEM of at least three independent assays.
ND, not determined.
Bgt, bungarotoxin.

presence of an inactive racemate; (+)AnTx is 2.5 times more potent than racemic AnTx [27]. While this will account for some of the disparity between (±)HomoAnTx and (+)AnTx, their respective potencies still differ by an order of magnitude. This contrasts with the full retention of potency at neuronal nicotinic binding sites, suggestive of the emergence of some sub-type selectivity.

Comparison with the agonist potency of other AnTx analogues in frog muscle [16] reveals that HomoAnTx is the most potent after (+)AnTx itself (with the exception of dihydroAnTx in which the azabicyclononene ring is saturated but the side-chain is unchanged). Thus, in those structures retaining the carbonyl group and conjugated diene, features shown to be crucial for agonist activity [16], removal of the side-chain methyl group or insertion of an ether oxygen were more detrimental to potency than the addition of a methylene group in the present structure.

We have previously demonstrated that N-methylation of any AnTx analogue results in a dramatic loss of both agonist potency and binding affinity [14, 16, 17, 29]. This trend was replicated in this study, in which *N*-methyl-HomoAnTx was a weak competitor of [^{3}H]nicotine binding to brain membranes (Table 1) and a very weak agonist in the muscle contracture assay (Fig. 3; Table 1). The N-methylated forms of cytisine, like AnTx, exhibit lower potency (see Ref. 30); in contrast N-methylation can increase the potency of some other nicotinic agonists, notably ferruginine, arecolone and carbamylcholine.

Molecular modelling studies of HomoAnTx [31] (Thompson *et al.*, in preparation) confirm that the extension of the carbon chain length does not result in a significant conformational distortion of the molecule relative to AnTx. In addition, it is clear that the planar extension of the carbon chain does not sterically hinder binding. The importance of this study is that it offers, for the first time, a route for the incorporation of tritium into a chemically stable site to generate a radiolabelled analogue of AnTx, with potency at neuronal nicotinic receptors comparable to that of the parent compound. Thus, HomoAnTx has the requisite characteristics of a potential tritiated agonist that would enable it to be exploited in the characterization of high affinity nicotinic sites in the brain. Moreover, the further extension of the carbon chain can be utilized to develop nicotinic affinity ligands [22]. In view of the burgeoning heterogeneity of nAChR subtypes in the nervous system [32], AnTx and its analogues emerge as potent and versatile probes.

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