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Solid-Phase Synthesis and Pharmacological Evaluation of Analogues of PhTX-12—A Potent and Selective Nicotinic Acetylcholine Receptor Antagonist

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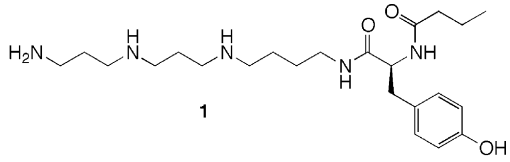
Abstract—Philanthotoxin-12 (PhTX-12) is a novel potent and selective, noncompetitive antagonist of nicotinic acetylcholine receptors (nAChRs). Homologues of PhTX-12 with 7–11 methylene groups between the primary amino group and the aromatic headgroup were synthesized using solid-phase methodology. In vitro electrophysiological studies of nAChR demonstrated that decreasing the number of methylene groups from 12 to 11 significantly increased potency. Antagonism by PhTX-11, like that of PhTX-12, was only weakly voltage-dependent. When the methylene spacer was reduced further, antagonism was decreased below that of PhTX-12, and in some cases potentiation of ACh responses by up to 60% was observed.

Polyamine toxins are a class of nonoligomeric, low molecular weight compounds isolated from the venom of spiders and wasps that are nonselective inhibitors of ionotropic receptors such as ionotropic glutamate receptors (iGluRs) and nicotinic acetylcholine receptors (nAChRs).[1] An example is philanthotoxin-433 (PhTX-433, **1**) isolated from the Egyptian digger wasp *Philanthus triangulum*.[2] PhTX-433 (**1**) and its synthetic analogue PhTX-343 (**2**) which are antagonists of a broad range of ionotropic receptors, show little selectivity toward these receptors.[1]

The polyamine moiety of polyamine toxins is considered to interact with polar or charged amino acid residues in the interior of cation-selective ion channels. However, it was recently demonstrated that by modification of the polyamine portion of **2** it was possible to achieve selectivity for the nAChR.[3] In particular, replacing the two secondary amino groups of **2** with methylene groups to give PhTX-12 (**3**) led to a dramatic increase of the antagonistic effect at nAChR, with a simultaneous loss of activity at various iGluRs.[3,4] Subsequent electrophysiological studies have shown that nAChR antagonism by **3** is only weakly voltage-dependent, contrary to antagonism by **2**. It was suggested that **3** antagonizes nAChR by enhancing desensitization, rather than by open-channel blockade, as is the case with **2**.[5] Given its high potency at nAChR and its selectivity for nAChR over ionotropic glutamate receptors, PhTX-12 (**3**) is a promising lead for the development of novel potent and selective noncompetitive antagonists of nAChR.



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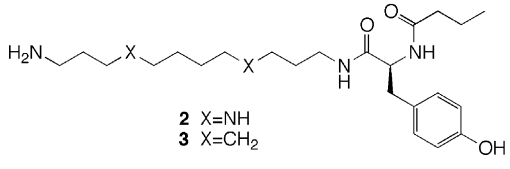

2 X=NH
3 X=CH_2

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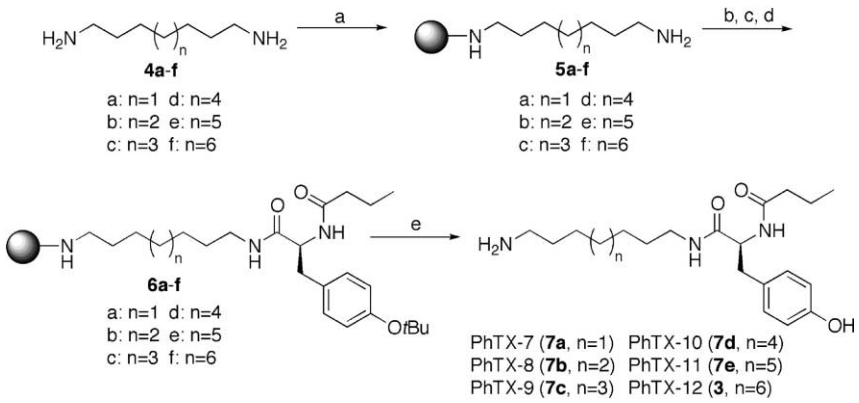

Scheme 1. (a) Trityl chloride resin; (b) (*S*)-Fmoc-*O*-(*tert*-butyl)tyrosine, HATU, collidine; (c) 20% piperidine; (d) butyric acid, HATU, collidine; (e) CH_2Cl_2/TFA/triisopropylsilane/H_2O (47.5:47.5:2.5:2.5).

To date, only limited structure–activity relationship (SAR) studies of **3** have been carried out, and these studies have shown that increasing the bulk and lipophilicity of the acyl portion of **3** decreases potency at nAChR,[6] in contrast to what was previously observed for **2**.[7] We report here the results of studies of the importance of the distance between the primary amino group and the aromatic head-group of **3**, for the antagonism of nAChR.

Chemistry

Solid-phase synthesis (SPS) has greatly facilitated the synthesis of polyamine toxins, in particular philanthotoxins.[1a] Tedious and often troublesome purification of highly polar intermediates encountered in classical solution-phase methods is replaced by simple washings when using SPS. Furthermore, the philanthotoxin molecule can be divided into three distinct parts (an amino acid moiety, an acyl group, and a long-chain polyamine moiety) linked by amide bonds. Thus, solid-phase methodology similar to that used in peptide synthesis can be applied for the synthesis of philanthotoxins, which are derived from symmetrical poly- or diamines.[1a,6]

The target compounds **7a**–**e** and **3** were synthesized utilizing SPS, as outlined in Scheme 1. Diamines **4a**–**f** were attached to trityl chloride resin to give **5a**–**f**. In order to minimize attachment to the resin of both amino groups of the diamine, a 10-fold molar excess of the diamines was used. Resins **5a**–**f** were derivatized using standard peptide coupling procedures. The first step was a reaction with (*S*)-Fmoc-*O*-(*tert*-butyl)tyrosine using HATU and collidine as coupling reagents. The Fmoc-protecting group was removed by treatment with 20% piperidine in DMF. Finally butyric acid was coupled to the free primary amino group to give resins **6a**–**f**. The final products were obtained by cleavage from the resins with concomitant deprotection of the phenol group, giving **7a**–**e** and **3** as crude products.

The major, and essentially the only impurity observed in the crude products was the starting diamine, originating from cross-linking of the resin. However, the toxins and the diamines could be easily separated using automated, preparative HPLC-MS.[8] The purified compounds were analyzed by HPLC-MS using simultaneous UV and evaporative light scattering detection (ELSD), the latter being capable of detecting the diamine impurities that are not observed by UV detection. The final purity of **7a**–**e** and **3** was in the range of 97–100% as assessed by the ELSD-trace. Since it was previously shown that coupling of (*S*)-Fmoc-*O*-(*tert*-butyl)tyrosine with the primary amine takes place without racemization of the former,[4] the products **7a**–**e** and **3** are assumed to be pure (*S*)-enantiomers.

In Vitro Electrophysiology

Whole-cell patch-clamp recordings were used to investigate the effects of PhTX-12 (**3**) and its homologues **7a**–**e** on human embryonic muscle-type nAChR which contains a δ-subunit rather than an ε-subunit present in adult muscle expressed in TE671 cells.[9] Cells were clamped at holding potentials (V_H) of +50, −50 and −100 mV, as previously described,[3,9] PhTX-12 (**3**) and homologues were co-applied with 10 μM acetylcholine (ACh). Concentration–inhibition curves were generated for each V_H, and IC_{50} values were derived from these curves (see Table 1, $V_H = -50$ and −100 mV).

Table 1. IC_{50} values for antagonism by PhTX-12 (**3**) and homologues on nAChR expressed in TE671 cells[a]

Compd	$IC_{50} \pm SE$ (μM) (number of cells)	
	$V_H = -50$ mV	$V_H = -100$ mV
PhTX-3	NT[b]	75.0 ± 4.2 (10)[c]
PhTX-7 (**7a**)	10.5 ± 1.5 (7)	5.44 ± 0.49 (7)
PhTX-8 (**7b**)	9.76 ± 0.52 (7)	5.03 ± 0.22 (6)
PhTX-9 (**7c**)	13.3 ± 1.6 (11)	15.1 ± 1.4 (5)
PhTX-10 (**7d**)	8.66 ± 1.79 (12)	~10 (6)
PhTX-11 (**7e**)	0.58 ± 0.10 (16)	0.46 ± 0.05 (10)
PhTX-12 (**3**)	1.53 ± 0.11 (23)	0.93 ± 0.09 (29)

[a]From decrease of the current elicited by 10 μM ACh at holding potentials (V_H) −50 and −100 mV by co-application of the antagonist.
[b]NT, not tested.
[c]From ref 10.

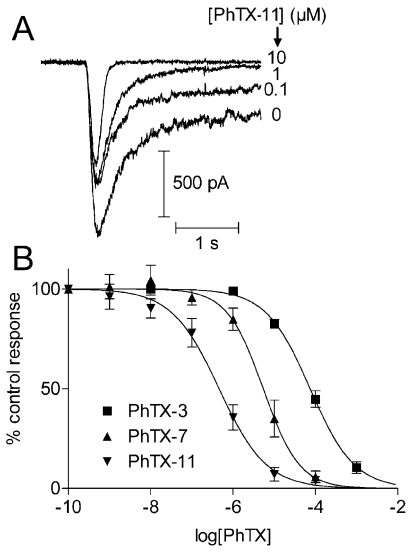

Figure 1. Inhibition of TE671 cell responses to ACh by PhTX-12 (**3**) homologues: (A) whole-cell currents at −100 mV evoked by application of ACh alone (0), or in the presence of 0.1, 1 and 10 μM PhTX-11 (**7e**); (B) concentration–inhibition curves for inhibition of responses to ACh by PhTX-11 (▼), PhTX-7 (▲) and PhTX-3 (■) at −100 mV.

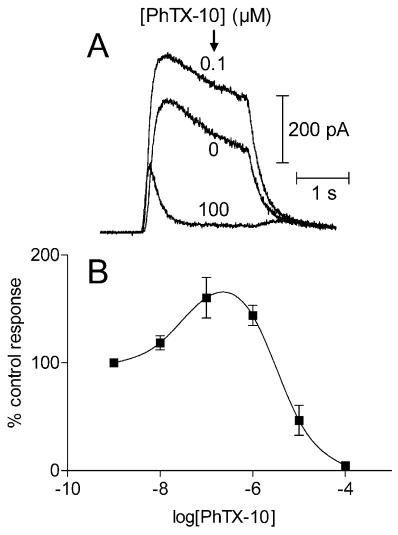

Figure 2. Potentiation and inhibition of responses to ACh of TE671 cells by PhTX-10 (**7d**): (A) whole-cell currents at +50 mV evoked by application of ACh alone (0), or in the presence of 0.1 and 100 μM PhTX-10 (**7d**); (B) concentration–potentiation/inhibition curve for the effect of PhTX-10 (**7d**) on responses to ACh at +50 mV.

PhTX-12 (**3**) has previously been shown to be a potent antagonist of nAChR[3] whereas PhTX-343 (**2**) is a less potent ($IC_{50}=16.6\pm0.24$ μM at −100 mV) voltage-dependent inhibitor of nAChR, and is essentially inactive at +50 mV.[5,10] In the present study, PhTX-12 (**3**) was a potent antagonist of nAChR with an IC_{50} value of 0.93 ± 0.09 μM (−100 mV) and was only slightly less potent at +50 mV, thus being almost voltage-independent.

Most of the homologues **7a**–**e** were less potent than PhTX-12 (**3**), with the exception of PhTX-11 (**7e**, Fig. 1). The latter was significantly more potent than **3** at both −50 and −100 mV, with an IC_{50} value of 0.46 ± 0.05 μM ($V_H=-100$ mV). Compounds PhTX-9 (**7c**) and PhTX-10 (**7d**) were both significantly less potent as antagonists than PhTX-12 (**3**), with IC_{50} values of 15.1 ± 1.4 and 10 μM ($V_H=-100$ mV), respectively. Interestingly, PhTX-7 (**7a**) and PhTX-8 (**7b**) were more potent than PhTX-9 (**7c**) and PhTX-10 (**7d**), with IC_{50} values of 5.44 ± 0.49 and 5.03 ± 0.22 μM, respectively, at −100 mV (Table 1). Finally, PhTX-3, with only three methylene groups between the primary amino group and the amide bond, was the least potent analogue ($IC_{50}=75.0\pm4.2$ μM at −100 mV).

Compounds **7a**–**e** were also examined at $V_H=+50$ mV (data not shown). At this V_H homologues **7a**–**d** had IC_{50} values in the range of 10–80 μM, showing a significant degree of voltage dependence. PhTX-11 (**7e**) had an IC_{50} value of 2.31 ± 0.44 μM, thus showing voltage-dependence similar to PhTX-12 (**3**). Finally, PhTX-10 (**7d**) caused a remarkable potentiation, by up to 60%, at concentrations below 3 μM at all three holding potentials (Fig. 2), while PhTX-8 (**7b**) and PhTX-9 (**7c**) potentiated ACh responses by up to 30% at concentrations below 10 μM at +50 mV.

Discussion and Conclusion

The importance of the length of the polymethylene spacer that separates the primary amino group from the aromatic head-group of PhTX-12 (**3**) was examined by synthesis of homologues **7a**–**e**, and subsequent characterization of their antagonistic effects at nAChR. It was shown that the 11 methylene spacer present in PhTX-11 (**7e**) significantly increased potency as compared to PhTX-12 (**3**), and showed a similar weak voltage-dependence. Reducing the length of the spacer further, as in **7a**–**d**, led to a significant decrease in potency, but the reductions in potency were not correlated to the number of methylene groups. Similarly, the voltage dependence of inhibition was not correlated with the number of methylene groups, although PhTX-7 (**7a**) and PhTX-8 (**7b**) were the most voltage-dependent. A greater voltage-dependent component of inhibition may explain the higher potency of **7a** and **7b** at −100 mV compared to PhTX-9 (**7c**) and PhTX-10 (**7d**).

Previous studies have suggested that PhTX-12 (**3**) antagonizes nAChR by enhancing desensitization rather than acting as an open channel blocker,[5] implying that **3** and PhTX-343 (**2**) binds at different sites on nAChR. The head-group of PhTX-343 (**2**) is believed to bind at the extracellular vestibule of the nAChR channel with the polyamine moiety binding inside the pore region, as indicated by photolabeling experiments.[11] In contrast, it has been suggested that PhTX-12 (**3**) binds to the outer ring of the pore,[5] probably to the binding site of a polyamine derivative, which causes voltage-independent inhibition of nAChR. This binding site has also been localized to the external vestibule of nAChR.[11b]

In the present study PhTX-11 (**7e**) and PhTX-12 (**3**) displayed very similar pharmacological profiles, although

7e is more potent than **3**. Antagonism of nAChR is reduced when the number of methylene in the spacer is 10 or less, showing that the primary amino group needs to be separated from the aromatic head-group by 11 or 12 methylene groups for maximum antagonistic activity at nAChR. However, **3** and **7e** are both very flexible molecules, and hence their pharmacologically active conformation is impossible to predict. Thus, further studies are required to clarify this point.

Homologues **7b–d** potentiated ACh responses at low concentrations, in the case of PhTX-10 (**7d**) by up to 60% (Fig. 2). A similar potentiation has previously been observed for spermine [*N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine] and PhTX-343 (**2**).[9] The potentiation is seemingly an inherent property of all the investigated compounds, and is probably caused by inhibition of ACh-induced desensitization;[5,9] however this effect is observed only if the antagonism of nAChR is sufficiently weak.

In summary, it was shown that PhTX-11 (**7e**) was significantly more potent than PhTX-12 (**3**), and showed similar weak voltage-dependence of inhibition. Homologues with shorter methylene spacer (**7a–d**) were significantly less potent than PhTX-12 (**3**) and at low concentrations they potentiated ACh responses, in the case of PhTX-10 (**7d**) by up to 60%.

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