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Binding of polyamine-containing toxins in the vestibule of the nicotinic acetylcholine receptor ion channel

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Abstract

Several wasp venoms contain philanthotoxins (PhTXs) that act as noncompetitive inhibitors (NCIs) on cation-selective ion channels including the nicotinic acetylcholine receptor (nAChR). In the search for a ligand with high affinity and specificity for the nAChR we tested a series of newly developed PhTX analogues. Modulation of the structural elements of PhTXs can significantly influence their binding affinities. This approach resulted in the development of the photolabile compound MR44. In photoaffinity labelling studies ^{125}I-MR44 was used to map the ligand-binding site at the *Torpedo californica* nAChR. Upon UV irradiation of the receptor–ligand complex, ^{125}I-MR44 was mainly incorporated into the receptor α-subunit. Proteolytic mapping and microsequencing identified the site of ^{125}I-MR44 cross-linking within the sequence αHis-186 to αLeu-199 that in its C-terminal region partially overlaps with the agonist-binding site. Since bound agonists had only minor influence on ^{125}I-MR44 photocross-linking, the site where the hydrophobic head group of ^{125}I-MR44 binds must be located outside the zone that is sterically influenced by agonists bound at the nAChR. A possible site of interaction of ^{125}I-MR44 would be the N-terminal region of the labelled sequence, in which aromatic amino-acid residues are accumulated. We suggest that the polyamine moiety of ^{125}I-MR44 interacts with the high affinity non-competitive inhibitor site deep in the ion channel, while the aromatic ring of this compound binds in the vestibule of the nAChR to a hydrophobic region on the α-subunit that is located close to the agonist binding site.

Keywords: Polyamine; Nicotinic acetylcholine receptor; Photoaffinity labelling; Ligand binding site

1. Introduction

The nicotinic acetylcholine receptor (nAChR) belongs to the superfamily of ligand-gated ion channels and is an integral transmembrane protein with a subunit stoichiometry of $\alpha_2\beta\gamma\delta$ [1–3]. The five receptor subunits are arranged around a central pore that opens for cations upon agonist binding. The primary structure of each subunit contains four sequences M1–M4 of particular hydrophobicity, which are long enough to traverse the plasma membrane [4]. The M2 sequences from all subunits have been shown to contribute to the formation of the ion pore [5]. The selectivity filter for cations is formed by several rings of negatively charged amino acid side chains protruding into the lumen of the pore [6–8].

Two binding sites for agonists and competitive antagonists are located in the extracellular region, mainly on the two α subunits [1] at the α–δ and α–γ interfaces [9,10]. A binding site for non-competitive inhibitors (NCIs), such as triphenylmethylphosphonium ($TPMP^+$), has been found in the lumen of the ion channel close to the M2 transmembrane domain [11,12]. Luminal NCIs are assumed to enter the open channel and to bind to different rings within the selectivity filter, thereby inhibiting the ion conductance by sterically plugging the channel pore [13].

The digger wasp *Philanthus triangulum* contains philanthotoxins (PhTXs) as active ingredients in its venom [14,15]. These natural polyamine amides of low molecular weight carry a hydrophobic head group that is

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linked to a positively charged polyamine tail. PhTXs are known to interact with cation-selective ion channels including the nAChR [16–18]. In previous studies it was shown that alteration of the structural elements of PhTXs could influence their binding affinity [19,20].

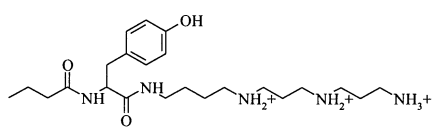
We have synthesized a series of novel polyamine-containing analogues of PhTX-433 (Fig. 1A) in a search for a ligand with high affinity and specificity at the *Torpedo californica* nAChR for photocross-linking studies. This approach resulted in the development of a photo-activatable compound, MR44 (Fig. 1B) [21] that was used to map the ligand-binding site in the lumen of the ion channel of nAChR [22].

2. Determination of binding affinities at the nAChR

To determine binding affinities of various newly developed PhTX derivatives at the nAChR, the fluorescent NCI ethidium was used in a displacement assay. Ethidium, when bound to the high-affinity NCI site on the nAChR in its desensitized state, shows an intensive emission maximum at 590 nm [23]. Since bound ethidium was displaceable by well-characterized luminal NCIs, such as $TPMP^+$ and chlorpromazine [23,24], ethidium can be used as a reference fluorophor to characterize yet unknown NCIs competing for this binding site.

To develop a photolabile ligand with high affinity and specificity for the nAChR, we varied the structural elements of the lead compound PhTX-433. From previous studies it is known that changes in the hydrophobic head group or the polyamine tail have major influence on the binding properties of these molecules [19,20,24]. Increasing the size of the hydrophobic head group of the PhTXs by introducing a bulky aromatic ring system or coupling decanoic acid significantly improved the binding affinity [20,24]. Polyamine analogues missing a hydrophobic head group were not able to displace bound ethidium, demonstrating that this hydrophobic element is essential for binding of these compounds to the nAChR NCI site [24]. Accordingly, the hydrophobic head group of PhTXs seems to be an important structural element that contributes significantly to high-affinity binding of these compounds. PhTX analogues with elongated polyamine tails, such as MR44, showed higher binding affinity, whereas a reduction in length resulted in a loss of binding. In general, symmetric polyamine analogues carrying a hydrophobic head group at both ends of the polyamine chain bound with higher affinity than their asymmetric analogues [24]. Remarkably, when introducing stiff aromatic elements or flexibly restricted macrocyclic systems into the polyamine chain the binding properties of these derivatives were significantly increased.

A



B

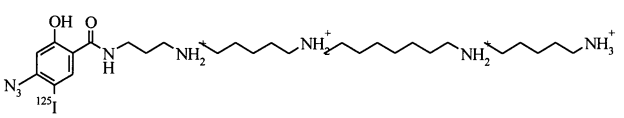


Fig. 1. Chemical formulae of PhTX-433 (A) and ^{125}I-MR44 (B).

3. Mapping the polyamine-binding site on the nAChR using ^{125}I-MR44 as a photoaffinity label

In binding studies the novel photolabile PhTX derivative ^{125}I-MR44 (Fig. 1B) was found to bind in the lumen of the nAChR ion channel to a single class of non-interacting binding sites. The binding stoichiometry was found to be two molecules of ^{125}I-MR44 per receptor monomer. Bound ^{125}I-MR44 was displaceable by well-characterized luminal NCIs, but not by agonist or competitive antagonists [22].

To localize the ligand-binding site, ^{125}I-MR44 was photocross-linked to nAChR-rich membranes and the region that incorporated ^{125}I-MR44 was mapped by proteolytic cleavage using V8 and AspN proteases [22]. The peptides generated were N-terminally sequenced and localized in the known primary structure of the *Torpedo* nAChR. After irradiation of receptor-bound ^{125}I-MR44, the photolabile PhTX derivative was photoincorporated into the receptor α-subunit. The site of ^{125}I-MR44 cross-linking was localized to a 14 amino-acid stretch (residues 186–199) [22] that overlaps in its C-terminal region with the agonist-binding domain [25–28]. Since the agonist carbachol or the competitive antagonist α-bungarotoxin had only minor influence on the photocross-linking yield of ^{125}I-MR44 [22], the site of interaction between the aromatic ring of ^{125}I-MR44 and the nAChR should be found outside the zone that is sterically influenced by any bound agonist contacting αTyr-190, αCys-192, αCys-193 and αTyr-198 [25–28]. Therefore, the site of interaction of the aromatic head group of ^{125}I-MR44 lies presumably within the hydrophobic sequence HWVY (residues 186–189) containing two aromatic amino-acid residues.

The photolabile group of ^{125}I-MR44 is located at its hydrophobic head, allowing the identification of the binding region of this part of the molecule. However, the site of interaction of the positively charged polyamine tail is less exactly determined by photoaffinity labelling. In back titration experiments,

MR44 was found to be displaceable by luminal NCIs, indicating that the site of MR44 overlaps with the high affinity-binding site deep in the channel lumen close to the selectivity filter [24]. This finding shows that the polyamine chain of ^{125}I-MR44 is oriented towards the narrow part of the ion channel, as also suggested by previous studies, while the hydrophobic head group of the molecule binds to a hydrophobic sequence that is located close to the agonist-binding site [22].

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