

# Effect of polyamines on the nicotinic ACh receptor

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## Abstract

**Introduction.** In recent years, interactions of various polyamines with a number of ionotropic receptors have been reported. Such interactions can be either negative (inhibition) or positive (potentiation). It is proposed that hydrophilic polyamines act as open-channel blockers and bind sites deeply in the ion channel pore. Hydrophobic polyamines are believed to act in the shallower part of the pore. There has been cause to think that polyamines with two aromatic moieties block the nicotinic acetylcholine (nACh) receptor by adopting a U-shaped conformation, that is, a conformation in which the long positively charged polyamine chain enters the ion channel while aromatic moieties interact with extracellular parts of  $\alpha$ -subunits.

**Objective.** Our goal was to determine whether and how changes in the structure of methoctramine (a polyamine with two aromatic moieties) affect the way in which the nACh receptor is blocked. We synthesized derivatives of methoctramine which have a less flexible structure than methoctramine itself and may be less capable of adopting a U-shaped conformation within the ion channel.

**Materials and method.** Whole-cell ACh-induced currents were recorded from mouse i28 satellite cells expanded in culture. Recordings were performed both in the presence and in the absence of polyamines.

**Results.** All tested polyamines applied at a concentration of 5  $\mu$ M blocked ACh-induced currents. Depending on the number of protonated nitrogen atoms, polyamines decreased the current amplitude and/or increased the decay rate of the current.

**Conclusions.** We propose two possible mechanisms to explain the action of polyamines: desensitization, and displacement of agonist molecules from their binding sites. The impact of the number of protonated nitrogen atoms is discussed.

## Key words

nicotinic acetylcholine receptors; non-competitive blockers; ionotropic receptors

## INTRODUCTION

Polyamines are organic compounds having hydrophobic head(s) on a polyamine backbone. At physiological pH, some polyamines, depending on their structure, are hydrophilic (due to positively charged ammonium groups) or hydrophobic (due to the presence of aromatic moieties). Polyamines occur naturally; for example, spermine and spermidine affect DNA synthesis and gene expression, and philanthotoxin PhTX-433 is a potent paralyzing toxin found in the venoms of certain spiders and wasps.

In recent years, interactions of natural and synthetic polyamines with acetylcholine and glutamate receptors have been reported [1, 2, 3]. The nicotinic acetylcholine receptor (nAChR) is an integral membrane protein which mediates fast synaptic transmission at the skeletal neuromuscular junction and at neuronal synapses found throughout the central and peripheral nervous system. The kinetics of the nAChR channel can be modulated by a broad group of ligands [4]. Non-competitive blockers are able to suppress the channel's activity without affecting binding of the agonist. Competitive blockers bind at the agonist's activating site. Co-activators potentiate the receptor's activity induced by the agonist. Both transmembrane and extra-transmembrane

locations are proposed as modulatory sites different from the sites where ACh acts as an agonist.

Modulation of the nicotinic acetylcholine receptor by polyamines can be either negative (inhibition) or positive (potentiation) [5, 6, 7, 8]. It has been proposed that the different modes of action of polyamines result from the different location of sites at which they bind and different hydrophilic/hydrophobic properties of the polyamines [7, 9, 10]. It has been suggested that a hydrophilic analogue of philanthotoxin PhTX-343 acts as an open-channel blocker and binds deeply in the ion channel pore. The open-channel blocker mechanism was proposed based on the observation that PhTX-343 reduced the lifetime of the open channel. It was suggested that the location of a binding site within the transmembrane part of the channel explained the voltage dependency of the action of PhTX-343. Moreover, it was suggested that the part of the polyamine molecule which had access to the site was a hydrophilic tail, while the hydrophobic "head" of PhTX-343 was accommodated by the more hydrophobic extracellular region of the pore. The hydrophobic polyamine analogue PhTX-(12), with a more complex action (stabilizing a particular state of a receptor – desensitized, open or closed), acted in the shallower part of the pore. It was suggested that such sites are hydrophobic and, therefore, bind terminal aromatic head group(s) only [6]. It is possible that some analogues have access to both types of sites.

Previous reports suggest that polyamines with one aromatic moiety bind the nACh receptor on each  $\alpha$ -subunit

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(i.e. they have two binding sites per receptor). Polyamines with two aromatic moieties were suggested to block the receptor in a 1:1 stoichiometric ratio [10]. In such a case it was proposed that the polyamine binds in a U-shaped conformation: the long positively charged polyamine chain reaches the transmembrane part of the ion channel, while each of the two aromatic moieties interacts with extracellular parts of  $\alpha$ -subunits.

## OBJECTIVE

Our goal was to determine whether and how changes in the structure of methoctramine (a polyamine with two aromatic moieties) affect the blocking of the nACh receptor. We also wished to determine which elements in the polyamine structure are important for polyamines' blocking potency. Methoctramine is a hydrophilic polyamine and is a well-recognized potent antagonist of the muscarinic ACh receptor [11, 12]. In the present study, methoctramine was modified by inserting dipiperidine (ELP7, ELP10) or dianiline (ELP21, ELP24) moieties in place of the inner octamethylene chain (Fig. 1). The modification made the compounds less flexible and possibly less capable of adopting a U-shape within the ion channel. We reduced the number of protonated nitrogen atoms in ELP21 and ELP24, while the number of protonated atoms in ELP7 and ELP10 was the same as in methoctramine. The effects of the modified polyamines on the kinetics of the nACh receptor were then investigated using a whole-cell technique [13].

## MATERIALS AND METHOD

**Cell culture and electrophysiology.** Patch clamp experiments were made on mouse i28 satellite cells differentiated *in vitro* for 3–6 days [14]. The i28 cells were cultured as previously described [14] in DMEM supplemented with heat-inactivated foetal calf serum (20%), L-glutamine (2mM), penicillin (100 units/ml), and streptomycin (100g/ml). In order to induce cell differentiation, the medium was replaced 1 day after plating with DMEM supplemented with 2% horse serum and L-glutamine, penicillin, and streptomycin.

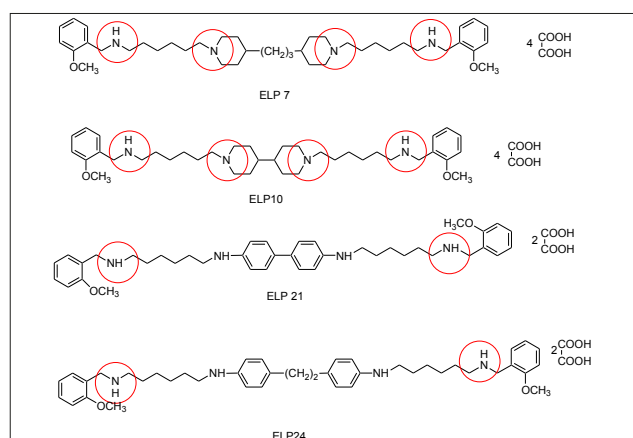
The bath saline (NES) contained (mM): NaCl 140, KCl 2.8, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 2, glucose 10, HEPES 10 (pH 7.4 with NaOH). The pipette contained: CsCl 140, MgCl<sub>2</sub> 2, HEPES 10, EGTA 0.5 (pH 7.3 with CsOH). Tested compounds (ACh and/or polyamines) were applied by gravitational perfusion system (RSC-200, Bio-Logic Rapid Solution Changer Perfusion). Solution exchange was performed by rotation of the rotating head of the RSC placed in a small distance to the tested cell. Pipettes resistances were 3–5 M $\Omega$ . Experiments were performed at –80 mV or –60 mV.

Polyamines were dissolved in NES (ELP7, ELP10, methoctramine) or in DMSO (ELP21, ELP24) and then incorporated in the perfusion solution. ACh-induced currents were recorded with Axopatch 200B (Axon Instruments, Foster City, CA, USA). The signals were filtered (2 kHz) and transferred to a hard disc. Whole-cell currents were analysed with pClamp 7 software (Axon Instruments).

Data are given as means  $\pm$  SE. One-sample *t*-test was used to determine statistical significance and Shapiro-Wilk test was used to assess the normality of data.

## Chemistry

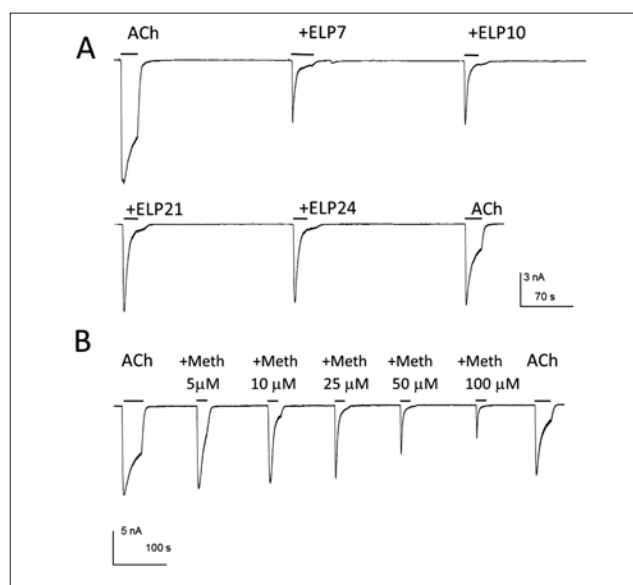
All the compounds were already described elsewhere [12]. Their spectral and physicochemical characterization (by IR, <sup>1</sup>H NMR, mass spectra, and elemental analysis) is given in the same article [12].



**Figure 1.** Structures of tested polyamines. Locations of protonated (at 7.4 pH) nitrogen atoms are marked with circles.

## RESULTS

Polyamines alone did not elicit any currents. ACh (5  $\mu$ M) induced a whole-cell current with fast activation and slow decay (Fig. 2). The current's amplitude  $I_{ACh}$  and its decay did not depend on DMSO (0.25%), used as a solvent for ELP21 and ELP24. The current decay represented desensitization of the receptor.

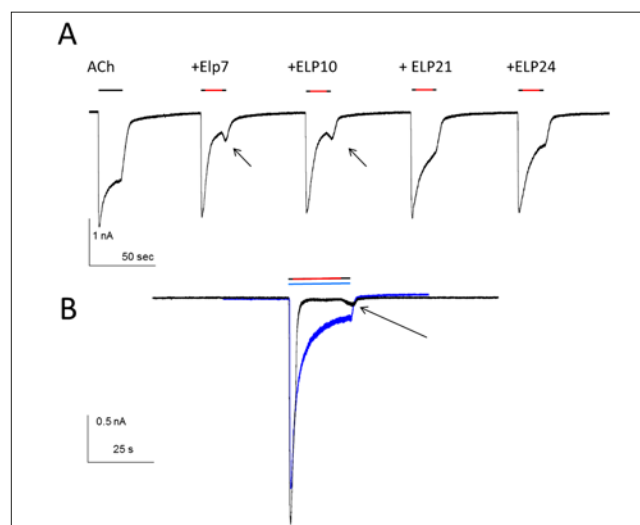


**Figure 2.** ACh-induced currents are reduced when ACh (5  $\mu$ M) is co-applied with polyamines. A) The current's amplitude decreased to about 50% in the presence of 5  $\mu$ M ELP7 and ELP10. ELP21 and ELP24 (5  $\mu$ M) did not affect the peak current. Time of decay increased in the presence of all polyamines. B) Different concentrations of methoctramine were applied. 5–100  $\mu$ M methoctramine accelerated current decay. Methoctramine decreased the current amplitude, except in the case of the lowest concentration (5  $\mu$ M). All blockers were applied simultaneously with the agonist.

ACh-induced current was reduced upon co-application of ACh with polyamines. This effect was not completely reversible after 100 sec washout, regardless of the polyamine structure.

In order to study the mechanism of polyamine blocking, we used different protocols for application of the agonist and polyamines. We observed that the effect exerted by ELP7 and ELP10 was different from the effect of ELP21 and ELP24. When ACh (5  $\mu$ M) was applied together with ELP7 or ELP10 (5  $\mu$ M), the current amplitudes were significantly smaller than the amplitudes elicited by ACh alone ( $I_{ACh}$  decreased by  $40.5\% \pm 4.8\%$ ,  $n=3$ ,  $p<0.02$  (ELP7) and by  $41.8\% \pm 3.2\%$ ,  $n=3$ ,  $p<0.02$  (ELP7); see Fig. 2A). ELP21 and ELP24 applied in the same concentration of 5  $\mu$ M did not significantly change the amplitudes of the current ( $I_{ACh}$  decreased by  $5.0\% \pm 2.8\%$ ,  $n=3$ ,  $p>0.2$  (ELP21) and by  $3.6\% \pm 0.9\%$ ,  $n=3$ ,  $p>0.05$  (ELP24); see Fig. 2AB). All polyamines accelerated the decay of the ACh current, but the extent depended on the blocker: the rate of decay increased about 2–3 times in the case of ELP7 and ELP10 and about 1.5 times in the case of ELP21 and ELP24 ( $n=3$ , Fig. 2A). To compare the effects of various ELPs with that of methoctramine, we performed two experiments in which we applied methoctramine (at different concentrations, 5–100  $\mu$ M) together with 5  $\mu$ M ACh. Methoctramine decreased the current amplitude and accelerated the current decay in a concentration-dependent manner (Fig. 2B). 5  $\mu$ M of methoctramine had a noticeable effect on the current decay (the rate of decay increased 1.5–2 times) and no significant effect on the current amplitude ( $I_{ACh}$  decreased by  $5.8\% \pm 2.2\%$ ,  $n=3$ ,  $p>0.1$ ).

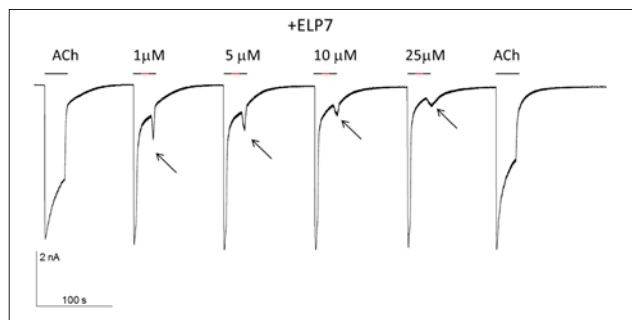
In another series of experiments, the blocker (after co-application with the agonist) was washed out before the agonist was removed (Fig. 3). The current recorded after the blocker had been removed depended on the polyamine structure. In the case of ELP7 and ELP10 (5  $\mu$ M) the current increased immediately after the blocker had been removed from the solution. We will use the term 'rebinding current' to describe this phenomenon. The amplitude of the 'rebinding current' was much lower than the amplitude of the current in the control recording (Fig. 3B).



**Figure 3 (A).** Current trace recorded during exposure to ACh alone (first peak) or in co-application with polyamine (5  $\mu$ M). A small fraction of the receptors in the presence of the agonist reopen quickly after the blocker is washed out, producing a 'reopening current' (ELP7 and ELP10 traces).

**B)** The amplitude of the 'reopening current' (black trace) is lower than that of the current induced by ACh alone (blue trace); ACh 5  $\mu$ M, ELP7 10  $\mu$ M; the arrows indicate a 'rebinding current'. Red lines indicate application of polyamine; black (or blue) lines indicate application of ACh.

To evaluate the dependency of ELP7 inhibition on the blocker concentration, 5  $\mu$ M ACh was co-applied with increasing concentrations of ELP7 (Fig. 4,  $n=2$ ). The higher was the concentration of the blocker, the greater was the degree of current blockage. The 'rebinding current' was observed for all studied concentrations of ELP7. The magnitude of the 'rebinding current' decreased when the concentration of the blocker increased.



**Figure 4.** Current trace recorded during exposure to 5  $\mu$ M ACh alone or together with ELP7. The arrows indicate a 'rebinding current'. Red lines indicate application of polyamine; black lines indicate application of ACh.

## DISCUSSION

All of the tested polyamines applied in a concentration of 5  $\mu$ M decreased the ACh-induced currents. In the case of ELP7 and ELP10, co-application of the polyamine with ACh (5  $\mu$ M) resulted in a decrease in the current's amplitude and acceleration of its decay (Fig. 2A). The effects of ELP21 and ELP24 were weaker; these molecules in a concentration of 5  $\mu$ M did not affect the current's amplitude and were less potent in accelerating its decay. This observation suggests that the quantity of protonated nitrogen atoms in the ELP molecule affects the blocker's potency: the potency increases with larger numbers of charged nitrogen atoms. The acceleration of the current decay suggests that polyamines increase the kinetic rate of a receptor's desensitization. The mechanism is common for a large number of allosteric blockers/modulators of the nACh receptor [7]. Furthermore, our observation that the quantity of charged nitrogen atoms affects the degree of desensitization of the receptors is consistent with previous studies showing that desensitization is regulated by electrostatic interactions [15].

In the following part of the Discussion, we will describe observations suggesting that polyamines ELP7 and ELP10 act according to two different mechanisms, only one of which involves desensitization of the receptor. Fig. 3 demonstrates that after washing out of ELP7 and ELP10, a fraction of the receptors reopen quickly (in the presence of ACh), producing a fast 'reopening current'. Since recovery from desensitization always occurs in the absence of the agonist, this 'reopening current' indicates that a fraction of the receptors are blocked by a mechanism other than their desensitization.

One possibility is that polyamines ELP7 and ELP10 act by the open-channel blocking mechanism. To verify this possibility, more experiments in single-channel configurations would be necessary. However, some features of the whole-cell currents are inconsistent with the predictions of the open-channel blocking theory. According to the theory, the open-channel blockers extend the duration of bursts and keep the total

open time per burst unchanged (the so-called 'total open time per burst paradox' [16]). In the case of a combination of two mechanisms, one preserving the total open time per burst (open-channel blocker mechanism) and one decreasing it (desensitization), the overall rate of current decay should be slower than in a situation where only one mechanism (desensitization) is present. We observed that the decay was faster in case of ELP7 and ELP10 than in case of ELP21 and ELP24. This observation suggests that ELP7 and ELP10 are not open-channel blockers. This possibility should be verified in single-channel experiments.

Even though it is necessary to perform more experiments to characterize in detail the polyamine-induced blocking mechanism that generates the 'reopening current', we propose that the phenomenon of a 'reopening current' is consistent with the mechanism proposed recently for blocking of the nACh receptor by hydrocortisone [17]. It was suggested that hydrocortisone was able to displace an agonist from its binding site. In this article, we propose that the actions of some polyamines (ELP7 and ELP10) may be regulated by a similar mechanism, i.e. displacement of the agonist from its binding site.

## CONCLUSIONS

In summary, we propose two possible mechanisms for the action of polyamines: desensitization and displacement. The results presented indicate that the blocking capacity of polyamines increases when the quantity of protonated nitrogen atoms is increased. Although the displacement mechanism requires verification by means of single-channel experiments, we would emphasize the fact that the amplitude of the 'reopening current' decreased as a result of higher concentration of ELP7 (Fig. 4). Therefore, in order to induce desensitization of the receptors, the concentration of polyamines needs to be higher than when the receptors are blocked by the mechanism producing the 'reopening current'. This means that polyamines may have different affinities in the two blocking mechanisms of desensitization and displacement.

## Authors' contributions

Concept of the study: VT; electrophysiology (cell culture, whole-cell experiments, data analysis): EN, BD; chemistry (ELPs synthesis, characterization by IR,<sup>1</sup>H NMR, mass spectra, and elemental analysis): VT; writing the manuscript: EN, BD.

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