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Natural polymeric 3-alkylpyridinium salt affects vertebrate skeletal muscle contractility by preferentially blocking neuromuscular transmission

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ABSTRACT

The effects of natural polymeric alkylpyridinium salt (nPoly-3-APS), a potent acetylcholinesterase inhibitor isolated from the marine sponge *Reniera sarai*, were studied on isolated mouse phrenic nerve-hemidiaphragm muscle preparations using electrophysiological approaches. nPoly-3-APS inhibited nerve-evoked isometric muscle twitch and tetanic contraction in a concentration-dependent manner (IC_{50} = 29.4 μM and 18.5 μM, respectively) and produced a 30–44% decrease of directly muscle-elicited twitch and tetanus amplitudes at 54.4 μM. Additionally, nPoly-3-APS (9.1–27.2 μM) markedly decreased the amplitude of miniature endplate potentials, while their frequency was only affected at the highest concentration used. Endplate potentials were also inhibited by nPoly-3-APS in a concentration-dependent manner (IC_{50} = 20.1 μM), without significant change in the resting membrane potential of muscle fibers (up to 54.4 μM). In conclusion, our results show, for the first time, that nPoly-3-APS preferentially blocks the neuromuscular transmission, *in vitro*, by a non-depolarizing mechanism. This strongly suggests that the *in vivo* toxicity of nPoly-3-APS mainly occurs through an antagonist action of the compound on nicotinic acetylcholine receptors of skeletal muscles.

1. Introduction

Natural polymeric 3-alkylpyridinium salt (nPoly-3-APS), isolated from the marine sponge *Reniera sarai* (Sepcic et al., 1997), shows a broad spectrum of biological activities, including antibacterial and antifouling activities, acetylcholinesterase (AChE) inhibition, cytotoxicity, as well as hemolysis and selective toxicity against non-small cell lung cancer (NSCLC) tumor cells *in vitro* and *in vivo* (Sepcic et al., 1997, 1998; Malovrh et al., 1999; Faimali et al., 2003; Tucker et al., 2003; Chelossi et al., 2006; Paleari et al., 2006; Catassi et al., 2008; Elersek et al., 2008). nPoly-3-APS can also be used for the stable transfection of nucleated mammalian cells (Tucker et al., 2003). Its likely application in medicine led to the synthesis of a series of analogs with different degree of polymerization and length of alkyl chains (Houssen et al., 2010). Synthetic analogs of Poly-3-APS, such as APS12-2, APS3 (Grandic et al., 2011, 2013), APS8 (unpublished data) and structurally related compounds, are promising new chemotherapeutic agents that exhibit very low *in vivo* toxicity in experimental animals. Recently, the effects of some of these synthetic analogs possessing potent AChE-inhibitory properties were studied on neuromuscular transmission in skeletal muscle (reviewed in Grandic and Frangez, 2014) to avoid unwanted peripheral side effects that could appear in patients treated with some AChE inhibitors. APS12-2 acts as a potent non-competitive AChE inhibitor with an inhibitory constant (K_i) of 34×10^{-3} nM (Houssen et al., 2010). It exerts hemolytic, antibacterial and antifungal actions (Houssen et al., 2010). Some nicotinic cholinergic antagonists, such as APS3 and APS8, have shown promise in lung cancer treatment because of their selective cytotoxicity against NSCLC, the most common form of lung cancer (Zovko et al., 2013). NSCLC cells express cholinergic signaling mediators, including neuronal-type α7-nicotinic acetylcholine receptors (Paleari et al., 2006; Catassi et al., 2008). The inhibitory effects of the synthetic analogs APS12-2 and APS-3 on neuromuscular transmission and nerve-evoked skeletal muscle contraction were previously reported (Grandic et al., 2012, 2013). It was demonstrated that the neuromuscular blockade produced by these two compounds is due to their direct inhibitory effect on muscle-type nicotinic acetylcholine receptors (nAChRs). Therefore, it was of interest to determine whether the natural compound nPoly-3-APS shares the same biological effects on neuromuscular transmission and skeletal muscle functioning. In particular, due to the structural similarity between nPoly-3-APS and

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quaternary ammonium compounds that have been reported to block muscle-type nAChRs, the aim of this study was to investigate the effects and underlying mechanisms of the natural compound on neuromuscular transmission and skeletal muscle contraction. We show, for the first time, that nPoly-3-APS affects the skeletal muscle functioning, *in vitro*, by preferentially blocking the neuromuscular transmission probably through an antagonist action on muscle-type nAChRs.

2. Materials and methods

2.1. Materials

2.1.1. Drugs

nPoly-3-APS was isolated from the marine sponge *Reniera sarai* according to Sepcic et al. (1997). Before use, the toxin was dissolved in a sterile 0.9% saline solution at a stock concentration of 10 mg/mL and was stored at 4 °C. Neostigmine methyl sulfate (Tokyo Chemical Industry CO./LTD, Japan), 3,4-diaminopyridine (3,4-DAP; Sigma-Aldrich, USA) and μ-conotoxin GIIIB (Bachem, Switzerland) were of the highest grade available.

2.1.2. Experimental animals and neuromuscular preparations

Adult male Balb/C mice were obtained from the animal breeding facility at the Veterinary Faculty (University of Ljubljana, Slovenia). All the experiments followed ethical standards and were approved by the administration of the Republic of Slovenia for food safety, veterinary and plant protection with permit no. 34401-20/2009/30.

Mice were sacrificed by cervical dislocation followed by immediate exsanguination. The diaphragm muscle along with both phrenic nerves was dissected and split into two neuromuscular preparations. Each hemidiaphragm was maintained in oxygenated standard Krebs-Ringer solution composed of the following: 154 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES and 11 mM D-glucose (pH 7.4). All experiments were performed at 22–24 °C.

2.2. Methods

2.2.1. Muscle twitch and tetanus recordings

The hemidiaphragm was pinned to the organ bath on its lateral side. It was connected to an isometric mechano-electrical transducer (Grass Instruments, West Warwick, RI, USA) by silky thread *via* a stainless-steel hook, attached to its tendinous part. The motor nerve of the isolated neuromuscular preparation was stimulated using a square pulse S-48 stimulator (Grass Instruments, West Warwick, RI, USA) *via* a suction electrode with pulses of 0.1 ms and supramaximal voltage (typically 5–10 V) applied at a 0.1-Hz frequency. Direct muscle stimulation was evoked by electric field stimulation *via* a platinum electrode assembly in the organ bath connected to the S-48 Grass stimulator and producing pulses of 0.1 ms and 50–120 V applied at a 0.1-Hz frequency. Directly- (*via* muscle stimulation) or indirectly- (*via* nerve stimulation) evoked tetanic contraction was obtained with a pulse train duration of 1000 ms and a stimulation rate of 80 Hz. To achieve the maximal contractile response upon nerve-evoked stimulation, the stable resting tension for each neuromuscular preparation (typically 1.5–2.5 g) was adjusted approximately 20 min before starting the experiment. Muscle twitch or tetanic tension was measured using a Grass FT03 force transducer (Grass Instruments, West Warwick, RI, USA). Electrical signals were amplified using a P122 strain gauge amplifier (Grass Instruments, West Warwick, RI, USA) and digitized using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA) at a sampling rate of 1 kHz. The inhibitory response of nPoly-3-APS was continuously measured during 60–90 min after compound application. The muscle twitch or tetanic tension blockade produced by nPoly-3-APS was expressed as the percentage of the maximal response recorded before compound application. The concentrations of nPoly-3-APS used were 9.1, 18.1, 27.2, 36.2, 45.3 and 54.4 μM.

2.2.2. Membrane potential recordings

Hemidiaphragm preparations were equilibrated for 30 min in standard Krebs-Ringer solution added with 1.6 μM μ-conotoxin GIIIB, which blocks $Na_V1.4$ muscle sodium channel subtypes (Cruz et al., 1985; Hong and Chang, 1989) and allows the recording of the full-sized amplitude of the endplate potentials (EPPs). Miniature endplate potentials (MEPPs), EPPs and the resting membrane potential (rV_m) were recorded from superficial hemidiaphragm muscle fibers using intracellular borosilicate glass microelectrodes and Axoclamp 900A microelectrode amplifier (Molecular Devices, Sunnyvale, CA, USA). Microelectrodes were pulled using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA) and were filled with 3 M KCl solution. Only microelectrodes with resistance of 10–20 MΩ were used. Endplate regions, where EPPs were recorded, were indicated by the presence of MEPPs. EPPs were evoked by stimulating the phrenic nerve *via* the bipolar suction electrode with supramaximal square pulses of 0.1 ms at 0.5 Hz using the S-48 Grass stimulator. MEPPs and EPPs were digitized at 25 kHz using Digidata 1440A and pCLAMP 10 (Molecular Devices, Sunnyvale, CA, USA) and were stored for later analysis using pCLAMP-Clampfit 10 software (Molecular Devices, Union City, CA, USA). The recordings were performed before treatment, 45 and 90 min after the application of nPoly-3-APS and 15 min after the compound wash-out. MEPP and EPP amplitudes were normalized to a rV_m of −70 mV using the formula $V_c = V_o \times (-70)/rV_m$, where V_c is the normalized amplitude of MEPPs and EPPs, and V_o is their recorded amplitude (Pardo et al., 2006). The concentrations of nPoly-3-APS used were 1.8, 9.1, 18.1, 27.2, 36.2 and 54.4 μM.

2.2.3. Data analysis and statistics

The results are presented as the means ± S.E. The nPoly-3-APS concentrations producing 50% inhibition of initial responses (IC_{50}) were determined by fitting the concentration-response relationships using the four-parameter nonlinear regression model (GraphPad Prism version 6.00). The data were statistically analyzed using Sigma Plot for Windows version 12.5 (Systat Software Inc., USA). Student's two-tailed *t*-test was used for statistical analysis of the data. Statistical significance was set at a P value ≤ 0.05.

3. Results

3.1. nPoly-3-APS blocks muscle contraction in vitro

The effects of natural nPoly-3-APS (9.1–54.4 μM) were first determined on nerve-evoked and directly muscle-elicited isometric twitch and tetanic contraction in mouse hemidiaphragm preparations. A representative example of the time-course of nPoly-3-APS effects on muscle contraction is shown in Fig. 1A. The compound (36.2 μM) blocked nerve-evoked twitch and tetanic contraction within 90 min (Fig. 1A and B). This effect was in part reversible since, after about 30 min of thorough washing of the preparation with standard physiological solution, the nerve-evoked muscle twitch and tetanic contraction were restored partially (Fig. 1A). In contrast, directly muscle-elicited twitch and tetanic contraction were much less affected by a similar compound concentration (Fig. 1A and C). The nPoly-3-APS-induced block of muscle contraction occurred in a concentration-dependent manner (Fig. 1D and E). The IC_{50} values, calculated from the concentration-response curves, were 18.5–29.4 μM for nerve-evoked muscle contraction, and 56–90 μM for directly muscle-elicited contraction (Fig. 1E). Therefore, nPoly-3-APS was more than 3 times more efficient to block nerve-evoked than directly muscle-elicited twitch and tetanic contraction. It is worth noting that no decrease of muscle contraction amplitude occurred during 90-min control experiments during which neuromuscular preparations were only bathed with Krebs-Ringer solution (data not shown).

During the development of the contraction block, obtained 30 min after the application of 36.2 μM nPoly-3-APS (*i.e.* 33 ± 4.4%, n = 4),