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Electrophysiological investigation of the effect of structurally different bispyridinium non-oxime compounds on human α7-nicotinic acetylcholine receptor activity—An *in vitro* structure-activity analysis

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ABSTRACT

Organophosphorus compounds, including nerve agents and pesticides, exert their toxicity through irreversible inhibition of acetylcholinesterase (AChE) resulting in an accumulation of acetylcholine and functional impairment of muscarinic and nicotinic acetylcholine receptors. Current therapy comprises oximes to reactivate AChE and atropine to antagonize effects induced by muscarinic acetylcholine receptors. Nicotinic malfunction leading to depression of the central and peripheral respiratory system is not directly treated calling for alternative therapeutic interventions. In the present study, we investigated the electrophysiological properties of the human nAChR subtype α7 (hα7-nAChR) and the functional effect of the 4-*tert*-butyl bispyridinium (BP) compound MB327 and of a series of novel substituted bispyridinium compounds on the receptors by an automated patch clamp technique. Activation of hα7-nAChRs was induced by nicotine and acetylcholine demonstrating rapid cationic influx up to 100 µM. Agonist-induced currents decayed within a few milliseconds revealing fast desensitization of the receptors. Application of higher agonist concentrations led to a decline of current amplitudes which seemed to be due to increasing receptor desensitization. When 100 µM of agonist was coapplied with low concentrations of the well characterized α7-specific positive allosteric modulator PNU-120596 (1 µM–10 µM), the maximum response and duration of nAChR activation were markedly augmented indicating an elongated mean open-time of receptors and prevention of receptor desensitization. However, co-application of increasing PNU-120596 concentrations (> 10 µM) with agonist induced a decline of potentiated current responses. Although less pronounced than PNU-120596, six of the twenty tested substituted BP compounds, in particular those with a substituent at 3-position and 4-position at the pyridinium moieties, were found to potentiate current responses of hα7-nAChRs, most pronounced MB327.This effect was clearly depended on the presence of the agonist indicating a positive allosteric mechanism of these compounds. Besides potentiation at low concentrations, these compounds seem to interact at different binding sites on hα7-nAChRs since enhancement decreased at high concentrations.

The residual fourteen BP compounds, possessing either an isopropyl-group or more than one group at the pyridinium moiety, antagonized nicotinic currents exhibiting IC_{50} of low up to high micromolar concentrations (~ 1 µM–300 µM).

1. Introduction

Organophosphorus (OP) compounds have been repeatedly used as chemical warfare agents, most recently the nerve agent sarin in Syria in 2013 (Pita and Domingo, 2014) and 2017 (OPCW, 2017) and are still posing a serious threat to military personnel and to civilian population (Wiener and Hoffman, 2004). These agentś acute toxic effects are based on irreversible inhibition of acetylcholinesterase (AChE, E.C.3.1.1.7), leading to an accumulation of the neurotransmitter acetylcholine (ACh) and subsequent dysfunction of both, muscarinic (mAChRs) and

Abbreviations: AChE, acetylcholinesterase; ACh, acetylcholine; BP, bispyridinium; CNS, central nervous system; I^-, iodide; mAChR(s), muscarinic acetylcholine receptor(s); MLA, methyllycaconitine citrate; nAChR(s), nicotinic acetylcholine receptor(s); OP, organophosphorus compound; PAM, positive allosteric modulator; PNS, peripheral nervous system; PNU-120596, N-(5-chloro-2,4-dimethoxyphenyl)-N'-(5-methyl-3-isoxazolyl)-urea; TfO^-, trifluoromethanesulfonate

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nicotinic receptors (nAChRs) (Holmstedt, 1959; Stewart, 1959; Koelle, 1963). This dysfunction comprises initial overstimulation of both cholinergic receptors and subsequent desensitization of nAChRs in case of continued overstimulation. The conventional treatment of OP intoxications comprises administration of atropine to antagonize the effects of elevated ACh levels at mAChRs and an oxime, such as obidoxime, to reactivate OP-inhibited AChE (Wilson and Ginsburg, 1955; Eyer and Worek, 2007; McDonough and Shih, 2007). In this context, overstimulation of nAChRs leading to desensitization of the receptors is not directly treated by the current therapeutic strategy. In this regard, nAChR desensitization is only reduced indirectly via ACh hydrolysis by oxime-restored AChE function. If untreated, desensitization of nAChRs may ultimately cause failure of the cholinergic neurotransmission including paralysis, seizures and loss of the central and peripheral respiratory drive (Grob and Harvey, 1953 Wright, 1954). An additional therapeutic challenge occurs with AChE inhibited by certain OP, such as tabun and soman, due to the rapid dealkylation of inhibited AChE that is resistant to therapeutic reversal by oxime (e.g. soman) and the formation of a reactivation-inaccessible AChE-OP-complex (e.g. tabun) (Fleisher and Harris, 1965; Worek et al., 2004, 2007). In order to overcome this therapeutic limitation, a direct interaction of pharmacologically active nAChR ligands preventing and recovering receptor desensitization was proposed as a new approach to counteract nerve agent poisoning more efficiently (Sheridan et al., 2005). In particular, ligands acting as positive allosteric modulators (PAMs) of nAChRs represent an important class of potential therapeutic drugs as they bind to a site distinct from the orthosteric binding site of ACh and thus may prevent nAChR desensitization by stabilizing its open state configuration (Edelstein and Changeux, 1998; Bertrand and Gopalakrishnan, 2007). Much of the recent work concerning nAChR modulators has focused on the homomeric α7-nAChR, which received considerable attention as drug target to treat cognitive and attention disorders underlying neuropsychiatric and neurodegenerative diseases (Léna and Changeux, 1998; Gotti and Clementi, 2004). By virtue of its high expression levels in the human body and its pharmacological profile including rapid desensitization by overstimulation being crucial in OP poisoning, the hα7-nAChR represents an important drug target mediating central symptoms after OP poisoning (Albuquerque et al., 1997; Sheridan et al., 2005; Fagerlund and Eriksson, 2009). Promising *in vitro* and *in vivo* studies (Tattersall, 1993; Turner et al., 2011; Seeger et al., 2012; Kassa et al., 2016; Price et al., 2016) demonstrated a positive pharmacological effect of the 4-*tert*-butyl bispyridinium (BP) compound MB327, which was partly attributed to its interaction with nAChRs at an allosteric site (Niessen et al., 2011, 2013, 2016). In the present electrophysiological *in vitro* study, a series of novel BP non-oximes were investigated for their ability to act as PAMs at hα7-nAChRs using an automated whole-cell patch clamp system. Thereby, CHO cells were used as a host system for stable transfection and expression of hα7-nAChR. Prior to investigation of the effect of BP compounds on hα7-nAChR, the specific expression of functional α7-nAChR in CHO cells was verified by elucidating the functional effect of the well-described α7-specific PAM PNU-120596 on hα7-nAChR. To this end, this work serves to identify structural requirements of substituted BP compounds mediating a positive pharmacological effect in order to find promising structures capable to treat nAChR desensitization after OP poisoning. Furthermore, identification of binding sites and receptor subtype selectivity of such compounds are further important future considerations for the development of compounds capable to efficiently prevent and recover desensitization.

2. Materials and methods

Cells derived from a Chinese hamster ovary cell line (*Cricetulus griseus*), CHO, stably expressing the hα7-nAChR (CHO/RIC-3/hα7-nAChR cell line) were obtained from Genionics (Schlieren, Switzerland). Cell culture media and supplements were purchased from Gibco, distributed by Invitrogen (Darmstadt, Germany). Acetylcholine iodide, nicotine and PNU-120596 were purchased from Sigma Aldrich (Taufkirchen, Germany). Methyllycaconitine citrate (MLA) was obtained from Tocris Bioscience (Wiesbaden-Nordenstadt, Germany).

Substituted bispyridinium compounds (also referred as MB327 and PTM substances) were synthesised by the Department of Pharmacy − Center for Drug Research, Ludwig-Maximillians-Universität München, Germany, and were available as iodide and triflate salts (Rappenglück et al., 2017a; Rappenglück et al., In preparation) (Fig. 6; Table 2 and 3).

All other chemicals were purchased from Merck Eurolab GmbH (Darmstadt, Germany) and from Carl Roth GmbH (Karlsruhe, Germany) at the purest grade available.

10 mM stock solutions of acetylcholine, nicotine and BP compounds were freshly prepared in external recording solution (see Section 2.2) and diluted to appropriate concentrations for electrophysiological measurements. PNU-120596 stock solutions (1 mM) were prepared in 10% aqueous DMSO and stored at − 80 °C until use.

2.1. Cell culture and harvesting

Stocks of stably transfected CHO/RIC-3/hα7-nAChR cells were maintained in Hams F12 medium (Gibco, distributed by Invitrogen, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C, 95% CO_2 and 90% humidity in an incubator and cultured continuously in the presence of 300 µg/ml hygromycin B to avoid outgrowth of cells that do not express hα7-nAChRs. The cells were harvested 2–3 days after cultivation (total cell passages 15–20), washed with PBS and detached from the surface by enzyme free dissociation buffer (TrypLE™Express, Gibco distributed by Invitrogen, Darmstadt, Germany). The cells were then centrifuged twice and the resulting cell pellet was resuspended in extracellular solution (see Section 2.2). Cell concentrations used for experiment were adjusted to 1×10^6 to 5×10^5 cells/ml.

2.2. Automated electrophysiological recordings

Membrane currents were recorded in a voltage-clamped whole-cell configuration performed with the Patchliner Octo® (Nanion Technologies, Munich, Germany), an automated patch clamp robot system, and conducted according to Nanion's standard procedure (Farre et al., 2009). The Patchliner Octo® is equipped with two EPC-10 Quadro patch clamp amplifiers (HEKA Elektronik, Lambrecht/Pfalz, Germany) for parallel current recordings of up to eight cells. Generation of voltage-clamp protocols and acquisition of data were carried out using the PatchControlHT software (Nanion Technologies, Munich, Germany) in combination with the PatchMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Current signals were filtered at 5 kHz before digitization and storage.

The electrolyte solutions had the following ionic composition: external solution contained 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH adjusted to 7.4 with NaOH; internal solution contained 50 mM KCl, 60 mM KF, 20 mM NaCl, 20 mM EGTA and 10 mM HEPES, adjusted to pH 7.4 with KOH. A seal enhancer solution containing 80 mM NaCl, 3 mM KCl, 10 mM $MgCl_2$, 35 mM $CaCl_2$ and 10 mM HEPES, pH 7.4 (HCl), was automatically applied to the external channel after cell capture in order to achieve better Gigaseals (> 1 Ω) and was replaced with external solution when the whole-cell configuration was established. All electrolyte solutions were filtered through 0.45 µM membrane (Merck Millipore, Darmstadt, Germany) and stored in aliquots at −20 °C.

2.3. Drug application

Current-voltage relations were determined by application of external solution for control as well as by agonist applications with and without 10 µM PNU-120596 and plotted against different holding