Toxicology Letters 247 (2016) 1–10

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Functional analysis of *Torpedo californica* nicotinic acetylcholine receptors in multiple activation states by SSM-based electrophysiology

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HIGHLIGHTS

- We developed a screening method for functional investigation of nicotinic acetylcholine receptors.
- We measured charge translocation via capacitive coupling using a SSM-based electrophysiology.
- We analysed the interaction of selected non-oxime bispyridinium compounds towards nicotinic acetylcholine receptors by selective induction of conformation shifts.

ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form 27 January 2016
Accepted 2 February 2016
Available online 3 February 2016

Keywords:
Nicotinic acetylcholine receptor
Torpedo californica
Activation states
Functional measurement
Solid supported membranes
Surface electronic event reader (SURFE^{2}R)

ABSTRACT

Organophosphorus compounds (OPC), i.e. nerve agents or pesticides, are highly toxic due to their strong inhibition potency against acetylcholinesterase (AChE). Inhibited AChE results in accumulation of acetylcholine in the synaptic cleft and thus the desensitisation of the nicotinic acetylcholine receptor (nAChR) in the postsynaptic membrane is provoked. Direct targeting of nAChR to reduce receptor desensitisation might be an alternative therapeutic approach. For drug discovery, functional properties of potent therapeutic candidates need to be investigated in addition to affinity properties. Solid supported membrane (SSM)-based electrophysiology is useful for functional characterisation of ligand-gated ion channels like nAChRs, as charge translocations via capacitive coupling of the supporting membrane can be measured. By varying the agonist (carbamoylcholine) concentration, different functional states of the nAChR were initiated. Using plasma membrane preparations obtained from *Torpedo californica* electric organ, functional properties of selected nAChR ligands and non-oxime bispyridinium compounds were investigated. Depending on overall-size, the bispyridinium compounds enhanced or inhibited cholinergic signals induced by 100 μM carbamoylcholine. Applying excessive concentrations of the agonist carbamoylcholine provoked desensitisation of the nAChRs, whereas addition of bispyridinium compounds bearing short alkyl linkers exhibited functional recovery of previously desensitised nAChRs. The results suggest that these non-oxime bispyridinium compounds possibly interacted with nAChR subtypes in a manner of a positive allosteric modulator (PAM). The described newly developed functional assay is a valuable tool for the assessment of functional properties of potential compounds such as nAChR modulating ligands, which might be a promising approach in the therapeutically treatment of OPC-poisonings.

Abbreviations: nAChR, nicotinic acetylcholine receptor; mAChR, muscarinic acetylcholine receptor; CNS, central nervous system; PNS, peripheral nervous system; OPC(s), organophosphorus compound(s); SURFE^{2}R, surface electronic event reader; SSM, solid supported membrane(s); NMG, *N*-methylglucamine; MB327, 1,1′-(propane-1,3-diyl) bis(4-*tert*-butylpyridinium) di(iodide); MB583, 1,1′-(ethane-1,2-diyl)bis(4-*tert*-butylpyridinium) di(iodide); MB781, 1,1′-(butane-1,4-diyl)bis(4-*tert*-butylpyridinium) di (iodide); MB779, 1,1′(decane-1,10-diyl)bis(pyridinium); PAM(s), positive allosteric modulator(s); Tris, tris(hydroxymethyl)aminomethane; Na^+_V, voltage-depended sodium channel(s).

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http://dx.doi.org/10.1016/j.toxlet.2016.02.002
0378-4274/

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are a heterogeneous family of pentameric ligand-gated cation channels (Unwin, 2013), which are ubiquitously expressed in neuronal and non-neuronal tissues. nAChRs are involved in a wide range of physiological and pathophysiological processes, like CNS disorders (e.g. Alzheimer's and Parkinson's disease, schizophrenia, depression, tobacco addiction) and PNS disorders (e.g. congenital myasthenic syndrome, myasthenia gravis) (Kalamida et al., 2007; Albuquerque et al., 2009). Consequently, drugs that modulate nAChR functions are becoming increasingly important (Gündisch, 2005; D'Hoedt and Bertrand, 2009; Hurst et al., 2013; Hogg and Bertrand, 2004). Furthermore, nAChRs are interesting therapeutic targets for the treatment of intoxications, e.g. by organophosphorus compounds (OPCs) (Albuquerque et al., 2006).

The toxic mechanism of OPCs such as nerve agents and pesticides is based on their irreversible inhibition of acetylcholinesterase (AChE, E.C. 3.1.1.7) (MacPhee-Quigley et al., 1985; Taylor et al., 1995), which results in overstimulation of both muscarinic and nicotinic acetylcholine receptors caused by accumulation of the neurotransmitter acetylcholine in the synaptic cleft, and may ultimately result in central and peripheral respiratory failure, seizures, and other signs of cholinergic syndromes (Marrs, 1993; Newmark, 2004). Standard treatment of OPC intoxication includes administration of a mAChR antagonist (atropine), an oxime (e.g. obidoxime or pralidoxime) to reactivate inhibited AChE, and a benzodiazepine (e.g. diazepam) to control seizures and convulsions (Worek et al., 2005; Eyer et al., 2007). AChE inhibited by certain OPCs is hardly reactivated by commonly used oxime AChE reactivators (e.g. tabun intoxication) or the enzyme-organophosphate complex undergoes a rapid dealkylation reaction (e.g. soman intoxication), thus preventing reactivation by oximes (Worek et al., 2004). In these cases, the disturbed neuromuscular transmission mediated by nAChR is therapeutically inaccessible. Therefore, peripheral respiratory arrest due to neuromuscular block is a major problem and calls for approaches to recover neuromuscular transmission at respiratory muscles (Thiermann et al., 2010).

Previous studies demonstrated that non-oxime bispyridinium compounds are able to induce the recovery of the neuromuscular transmission other than by classical reactivation of the inhibited AChE enzyme (Tattersall, 1993; Seeger et al., 2012). Depending on the linker length, bispyridinium compounds interact directly at nAChR by influencing the binding of the highly affine agonist [^{3}H] epibatidine (Niessen et al., 2013). Consequently, studying their functional effect are topics of interest.

nAChRs are allosteric proteins with dynamic interconversions between multiple conformational states, with the equilibria among these states regulated by ligand binding (Papke, 2014). Activation and deactivation of nAChRs is primarily controlled by the binding of agonists or antagonists at conventional agonist binding sites (orthosteric binding sites), but is also regulated by allosteric binding of modulatory acting substances. If no agonist is bound, the nAChRs are most stable in the resting (closed) state. With an agonist bound, the population of the open states increases. With regard to muscle-type nAChRs, the probability of short-lived openings and longer openings vary with the level of agonist binding, e.g. single binding, when agonist concentrations are low, or both binding sites, when agonist concentrations are higher (Williams et al., 2011). In presence of high agonist concentrations, an equilibration is reached, which will ultimately favour the desensitised state (Papke, 2014).

Therapeutically of interest are positive allosteric modulators (PAMs), which reduce or reverse nAChR desensitisation. PAM-based treatments are expected to augment the endogenous cholinergic tone in a spatially and temporally restricted manner (Uteshev, 2014).

In the present study, investigations on receptor interactions were performed with plasma membranes from the *Torpedo californica* electric organ, a rich source of the αβδg nAChR subtype localized at the neuromuscular junction (Whittaker, 1989). *Torpedo* nAChRs show a high degree of homology with human muscle-type nAChRs (Millar, 2003).

Functional measurements were carried out with electrophysiological measurements based on SSM, a rather new technique that detects charge translocations via supported membrane electrode (Schulz et al., 2008). In this technique, proteoliposomes or membrane vesicles are adsorbed onto an SSM. Integral proteins, e.g. ion pumps, transporters or ion channels are activated by a rapid substrate concentration jump. SSM-based electrophysiology is extremely useful in cases where conventional electrophysiology cannot be applied. In addition, SSM-based electrophysiology is attractive for screening application in drug discovery because of its robustness and potential for automation. In the recent past, SSM-based physiology has been used for the functional characterization of transporters (Ganea and Fendler, 2009) and ion channels such as nAChRs (Schulz et al., 2009).

In the present work, multiple activation states of the nAChR were analysed, and the interaction of selected compounds with them. In particular, the desensitised receptor state was topic of interest. As described previously (Arias et al., 2010, 2012, 2013), the desensitised state of nAChRs was manipulated *in vitro* by an excess of desensitisation-inducing compounds, i.e. carbamoylcholine. Due to rapid exchange of non-activating, activating and desensitising buffer, shifts into the conformations of resting, activated and desensitised state were forced, using the same sensor.

2. Material and methods

2.1. Materials

T. californica electroplaque tissue was purchased from Aquatic Research Consultants (San Pedro, CA, USA). Polypropylene microtiter plates, tubes and tips were from Eppendorf (Hamburg, Germany). Epibatidine, [5,6-cycloheptyl-^{3}H] with a specific activity of approximately 2 TBq/mmol and other disposables, such as filtermates and solid scintillators, were obtained from Perkin Elmer (Jügesheim, Germany). (±)-Epibatidine was purchased from Tocris (Bristol, UK) and pancuronium bromide, carbamoylcholine dichloride and (−)-nicotine hydrogen tartrate from Sigma–Aldrich (Taufkirchen, Germany). The tested bispyridinium compounds MB327, MB583, MB781, MB779 (MB compounds, chemical structures see Fig. 1) were synthesised at Dstl Porton Down, UK, and were ≥98% pure measured by NMR spectroscopy and mass spectrometry (Timperley et al., 2005). Stock solutions of MB compounds, pancuronium bromide, (−)-nicotine hydrogen tartrate and carbamoylcholine dichloride were prepared in distilled water (1 − 10 mM), and (±)-epibatidine in ethanol (1 mM).

2.2. Preparation of nAChR enriched plasma membrane fragments

Membranes from the frozen electric organ of *T. californica* were prepared and purified by a modified sucrose gradient density centrifugation as described before (Niessen et al., 2013). The obtained plasma membrane preparation in storage buffer (10 mM sodium phosphate buffer, 300 mM sucrose, 120 mM NaCl, 5 mM KCl, 0.5 mM EDTA, 0.1 mM PMSF (freshly added), 1 pill EDTA-free protease inhibitor combination per 50 ml ("Complete® EDTA free", Roche, Grenzach-Wyhlen); pH 7.4) was stored at −150 °C until use.