THE INTERACTION OF QUATERNARY REVERSIBLE ACETYLCHOLINESTERASE INHIBITORS WITH THE NICOTINIC RECEPTOR

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Short title: Acetylcholinesterase inhibitors influence nicotinic receptor

SUMMARY:

Background: Acetylcholinesterase inhibitors (AChEIs) are used in the treatment of myasthenia gravis (MG). We investigated the effects of AChEIs on peripheral nicotinic receptors (nAChR), which play a crucial role in the treatment of MG symptoms. The positive modulation of those receptors by AChE inhibitors could have an added value to the anti-AChE activity and might be useful in the therapy of MG. Furthermore, to estimate the potential drawbacks of the compounds, cytotoxicity has been assessed on various cell lines.

Methods: The whole-cell mode of the patch-clamp method was employed. The experiments were performed on medulloblastoma/rhabdomyosarcoma cell line TE671 expressing human embryonic muscle-like receptor with subunits $\alpha_2\beta\gamma\delta$. The effect of the compounds on cell viability was measured by standard MTT assay (Sigma Aldrich) on ACHN (renal cell adenocarcinoma), HeLa (immortal cell line derived from a cervical carcinoma), HEPG2 (hepatocellular carcinoma) and BJ (skin fibroblasts) cell lines.

Results: No positive modulation by the tested AChE inhibitors was observed. Moreover, the compounds exhibited antagonistic activity on the peripheral nAChR. Standard drugs used in MG treatment were shown to be less potent inhibitors of muscle-type nAChR than the newly synthesized compounds. The new compounds showed very little effect on cell viability, and toxicities were comparable to standards.

Conclusion: Newly synthesized AChEIs inhibited peripheral nAChR. Furthermore, the inhibition was higher than that of standards used for the treatment of MG. They could be used for the study of nAChR function, thanks to their high antagonizing potency and fast recovery of receptor activity after their removal. However, since no positive modulation was observed, the new compounds do not seem to be promising candidates for MG treatment, even though their cytotoxic effect was relatively low.

KEYWORDS: acetylcholinesterase inhibitor, nicotinic receptor, myasthenia gravis, patch-clamp, cytotoxicity

INTRODUCTION

Nicotinic acetylcholine receptors (nAChR) belong to a "Cys-loop" family of ligand-gated ion channels together with receptors for serotonin (5-HT3), gamma-aminobutyric acid (GABAA, GABAC) or glycine (Lester at al. 2004). nAChR are always formed from five subunits; however 17 distinct subunits have so far been identified (Broad et al. 2006; Colombo et al. 2013). These subunits can be differently combined so that they create a broad spectrum of nAChR, which vary in structure and pharmacology. nAChR can be classified by the localization in the organism. Muscle-type receptors are situated on the neuromuscular junctions and are composed of α_1, β_1, δ, ε or γ. The adult and fetal isoforms differ by the substitution of the γ by the ε subunit. The stoichiometry of the muscle type of receptor is always $(\alpha_1)_2\beta(\varepsilon)\gamma\delta$. The neuronal-type of receptor consists of either heteromeric (α_{2-6} and β_{2-4}) or homomeric (α_{7-10}) subunits (Broad et al. 2006).

Concerning physiological function, nAChR are involved in cholinergic synaptic transmission, playing an important role in physiological mechanism in both the peripheral and central nervous system. Alterations in their number and/or function are associated with many disorders including schizophrenia, epilepsy, myasthenia gravis, glaucoma and constipation, as well as neurodegenerative diseases such as Alzheimer´s and Parkinson´s (Pereira et al. 2002, Gotti and Clementi 2004). The treatment of such diseases relies on compounds, which are able to modulate nAChR function (for reviews see Astles et al. 2002, Gotti and Clementi 2004).

Acetylcholinesterase inhibitors (AChEIs) are used in the treatment of disorders with impaired cholinergic transmission. The inhibition of acetylcholinesterase (EC 3.1.1.7.; AChE) is a treatment strategy for early and mild type myasthenia gravis (MG), an autoimmune disease characterized by fatigable weakness of voluntary muscles. It is the result of post-synaptic membrane destruction in the neuromuscular junction (Turner 2007, Komloova et al 2011a, Kumar and Kaminski 2011). Currently, the peripherally-acting AChE inhibitors neostigmine and pyridostigmine are the most commonly used drugs. Peripheral action of those drugs is ensured by the structural motif represented by the quaternary nitrogen, which results in difficult penetration across the blood-brain-barrier (BBB). The benefit of

this localization is the lack of the central side effect; by the same token, however, these drugs cannot therefore be used as potential candidates for the treatment of Alzheimer's disease.

In our study, we have chosen two standard drugs: the clinically-used AChEI edrophonium and the experimentally-used selective AChEI BW284c51 (Fig.1) and the two most effective representatives, of different scaffold, from the new series of peripheral AChEI (K298, K524; Fig.1). The new peripherally acting AChEI were prepared as new potential candidates for the treatment of early-stage MG. Edrophonium chloride is used as a diagnostic tool for MG, because of its rapid onset and short-duration of pharmacological action (Scherer et al. 2005). BW274c51 (1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide) is a selective AChEI, and thus nonspecific butyrylcholinesterase (BChE) remains active for other substrates (Gorelick 1997). Based on the presumption that cholinesterase inhibitors can behave as nicotinic receptor modulators, especially those with a quaternary ammonium group (Olivera-Bravo et al. 2007), we investigated their effects on peripheral nAChRs, which play a crucial role in the treatment of MG. Novel bis-isoquinolinium (K298) and bis-quinolinium (K524) AChEIs were selected thanks to their high inhibitory potential (nanomolar range) and selectivity towards AChE, at least comparable to standard anti-MG drugs (Musilek, 2011; Komloova, 2011). Our hypothesis was that their positive modulation effect on nAChR could be an added value to their promising anti-AChE activity and might find use in the therapy of MG. Furthermore, any undesired potential antagonizing effect would be revealed. To assess the nicotinic profile, a patch-clamp study was conducted. Additionally, to estimate compounds´ potential toxicity, cytotoxicity has been assessed on various cell lines.

MATERIALS AND METHODS

Chemicals. K298 and K524 were synthesized at the Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic (Musilek, 2011; Komloova, 2011b). Purity (>99%) was assessed using TLC and HPLC methods as described previously.(Jun, 2007; Jun, 2008). All other reagents were purchased from Sigma Aldrich (Prague, Czech Republic).

Cell Culture and Maintenance. The experiments were performed on medulloblastoma/rhabdomyosarcoma cell line TE671 expressing human embryonic muscle-like receptor with subunits $\alpha_1\beta_1\gamma\delta$ (Schoepfer et al. 1988, Stratton et al. 1989), kindly provided by Dr. Jan Říčný. TE671 cells for the experiments were cultivated on coverslips at 37 °C under a 5 % CO_2 atmosphere in Dulbecco's Minimal Essential Medium (D-MEM) with 10% fetal bovine serum. Nicotine (100 μM) was added to the cultivation medium 2-3 days before the measurement to increase nicotinic receptor expression (Ke et al. 1998).

Whole–Cell Recordings. The whole-cell mode of the patch-clamp method was employed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA) as described previously (Soukup, 2011, Soukup 2013). Fire-polished borosilicate glass micropipettes with an outer diameter of approx. 3 μM were filled with a solution of the following composition (mM): CsF 110, CsCl 30, $MgCl_2$ 7, Na_2ATP 5, EGTA 2, HEPES-CsOH 10, pH 7.4. The resulting resistances of the microelectrodes were between 3 and 5 MΩ. The cell bath solution consisted of (mM): NaCl 160, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 2, HEPES-NaOH 10, glucose 10, pH 7.3 Solutions of compounds were applied using a rapid microperfusion system (Mayer et al., 1989) consisting of an array of ten parallel quartz-glass tubes, each approximately 400 μM in diameter. The tubes were positioned in the vicinity of the recorded cells and the flow of various solutions was switched on/off under microcomputer control (Dittert et al., 1998) A complete change of the solution around the cell could be carried out in 30 to 60 ms. Cells were held at -40mV during recording. For signal recordings and evaluation of data, an Axon Instruments Digidata 1440A digitizer and pCLAMP10 software package (Axon Instruments, Foster City, CA) were used.

Inhibition of peak current elicited at a given ACh concentration (I_{ACh}) by AChEIs. The application of 100 μM acetylcholine (ACh) was used as a control of the cell sensitivity. The solution of inhibitor was administered as a 5s pre-application followed by co-application together with 100 μM ACh.

ACh dose-response curve in the presence of AChEIs. ACh dose-response curves were obtained by application of ACh (10^{-7}-10^{-4}) either alone or together with each AChEI at its approximate IC_{50}.

Cell viability assessment

Standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Sigma Aldrich) was applied according to the manufacturer´s manual on ACHN (renal cell adenocarcinoma), HeLa (immortal cell line derived from a cervical carcinoma), HEPG2 (hepatocellular carcinoma) and BJ (skin fibroblasts) cell lines (ECACC, Salisbury, UK). The cells were cultured according to ECACC recommended conditions and seeded onto a clear-bottomed U-type 96-well plate (Vitrum, Czech Republic) at a density of 10,000 per well. Cells were exposed to the tested compounds for 24 hours, the medium was then replaced with a medium containing 10 μM of MTT, and the cells were allowed to produce formazan for another 1 hour. After that period, the medium containing MTT was sucked out and crystals of formazan remaining in the wells were dissolved in DMSO. Cell viability was assessed spectrophotometrically by the amount of formazan produced by mitochondrial oxidoreductases. Absorbance was measured at 570 nm with 650 nm reference wavelength on Synergy HT (BioTek, USA). IC_{50} was then calculated from the triplicates using non-linear regression (four parameters) by GraphPad Prism 5 software. The final IC_{50} value was obtained as the mean of at least 3 independent measurements. Due to limited hydrophilicity, the highest concentration of the tested compounds was set at 3mM. If the inhibition of cell viability did not reach 50% even at this highest concentration, then IC_{50} was regarded as higher than 3mM.

RESULTS

AChEI inhibition of nicotinic currents

The membrane potential was held at -40 mV. None of the tested newly synthesized AChEI exerted any effect on the total membrane conductance. Interestingly, edrophonium in higher concentrations induced minor ionic currents (Fig.2). However, when AChEI were pre-applied 5s before ACh, a significant I_{ACh} dose-dependent inhibition was observed. Fig. 3 shows the antagonistic effect of different AChEI concentrations (10^{-7}-10^{-4} M) on the I_{ACh} elicited by the application of 10^{-4} M of ACh. Normalized dose-response curves were fitted to a sigmoidal function and inhibitory potency expressed as IC_{50} was estimated (Table1). AChEIs produced reversible inhibition of nAChR, which was revealed

by subsequent application of ACh. The obtained data show that the standards used in MG treatment are less potent inhibitors of muscle type nAChR than the newly synthesized compounds.

Cytotoxicity evaluation on four different cell lines showed that the newly-prepared compounds exert a higher cytotoxic effect. However this effect can still be considered as very low, and in itself does not discriminate the compound from further development (Table 2).

ACh dose-response curve in the presence of inhibitors.

To better characterize AChEI effect on I_{ACh}, dose-response curves were obtained in both the presence and absence of AChEI. When the same ACh concentrations were co-applied with an approximately IC_{50} dose of AChEIs, I_{ACh} was decreased by about 50 % as compared to values obtained in the absence of inhibitors (Fig. 4). This effect was independent of the agonist concentration and reflects the non-competitive mechanism of the tested compounds. EC_{50} for ACh alone was 7 ± 0.9 μM, but in the presence of BW284c51 this value is increased to 8.7 ± 2.0 μM, and even higher for edrophonium (12.5 ± 1.5 μM). The newly-synthesized compounds reduced the affinity of ACh for nAChR approximately 3-fold. EC_{50} of ACh in the presence of K298 was 22.6 ± 1.5 μM and of K524 (1μM) was 21.8 ± 5 μM.

DISCUSSION

Nicotinic receptors are targets of many naturally occurring ligands. Our present study shows that quaternary ammonium compounds exert inhibitory effect on nAChR. However, similar findings have been reported previously for structurally different AChEI (Yost and Maestrone 1994, Olivera-Bravo et al. 2007). Based on our observations, the newly synthesized compounds (K298, K524) exert a non-competitive antagonism (Fig. 4) on the muscle-type nAChR expressed by the TE671 cell line. The acceleration of desensitization, as reported for edrophonium (Yost and Maestrone 1994), was not observed.

The novel compounds showed excellent *in vitro* AChE-inhibitory potency similar to that of AChEI standards used in the treatment of cholinergic deficits (Koomlova 2011b, Musilek 2011).

Unlike the others, edrophonium possesses just one quaternary nitrogen in its structure. Interestingly, only edrophonium from the tested group of compounds demonstrated minute active response, when applied alone. The maximal inhibitory effect of edrophonium on nAChR was reached at the concentration of 300 μM. However, our data showed that edrophonium is able to activate the receptor at a concentration of 300 μM and higher. Lower concentrations showed no direct effect, which is in accordance with previous data (Yost and Maestrone 1994). Thus it is possible that edrophonium acts as benzoquinonium, which activates and subsequently desensitizes the receptor (Arias 2000). Unsurprisingly, edrophonium showed lower affinity to the receptor than ACh. It is well known that some AChEIs (e.g. tacrine, physostigmine) cause inhibitory effects of the ACh response on AChR (Canti et al. 1998). Non-competitive inhibitory effect on nAChR caused by AChEIs can be explained by negative allosteric modulation (Hogg et al. 2005), by antagonism of ion channel opening or promoting desensitization (Lindovsky et al. 2012). In our experiment all the tested compounds showed completely reversible interaction and the similarity in the slopes of the dose-inhibition curves indicated that all of them could be fitted to a sigmoidal function. Hill coefficients are close to 1 representing the fact that their inhibition by binding to the nAChR is in 1:1 molecular ratio. Nevertheless, the differences in potency might be achieved by interaction with different sites on the receptor. Although both compound BW284c51 and edrophonium have an ability to block an open-channel, edrophonium seems to bind even more deeply within the channel (Olivera-Bravo et al. 2007) and displays one order of magnitude lower potency to inhibit the receptor. According ACh dose response curves of other tested bis-quaternary drugs is possible to exclude competitive mechanism. Neither elicited any agonistic effect on muscle-type nicotinic receptor. Thus, these compounds, rather than being promising candidates for MG treatment, could be interesting compounds for the prophylaxis of nerve agent poisoning (Soukup, 2013). Thus, these compounds may partially protect the AChE and treat the nicotinic overstimulation, since they are able to inhibit the ACh response more effectively within the increasing concentration of ACh.

In conclusion, drugs can positively affect nicotinic neurotransmission by four major mechanisms - by AChE inhibition, by inhibition of AChR, by sensitization of nicotinic AChR (positive

allosteric modulators), and finally by increasing the stability of the open-channel state (Maelicke and Albuquerque 2000). A combination of the AChE inhibition and the positive allosteric modulation would be beneficial for compounds intended for MG treatment. Unfortunately, the newly synthesized compounds fulfill only the first mechanism. Furthermore, their higher inhibitory efficacy to nAChR than edrophonium, used for the treatment of MG, makes them rather unpromising no matter that their toxic potential was relatively low. Therefore, in terms of the given potential clinical application (MG), the new compounds do not represent promising drugs. Unfortunately, neither potency nor clinically relevant effects were observed. Instead, the novel compounds could find use in the prophylaxis of nerve agent poisoning and in the study of nAChR function, thanks to their high inhibitory potency on AChE and on the peripheral nAChR, and the fast recovery after their removal.

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Table 1. Inhibitory potency of tested quaternary inhibitors.

Compound/ IC50 (nM)	**Edrophonium**	**BW284c51**	**K298**	**K524**
hAChE	5,200 ± 100[1]	30 ± 6[1]	5 ± 0.1[2]	1 ± 0.2[1]
nAChR	44,000 ± 5,000	5,600 ± 360	1,900 ± 200	570 ± 50

Values represent IC_{50} values with their ± S.E.M, hAChE stands for human acetylcholinesterase; nAChR for muscle type of nicotinic acetylcholine receptor expressed by TE671 cell line (n=6). [1]Musilek et al 2011; [2]Komloova et al 2011b.

Table 2. Cytotoxic effect of quaternary compounds

Compound/ IC50 (mM)	**Edrophonium**	**BW284c51**	**K298**	**K524**
ACHN	>3	>3	1.05 (0.64 – 1.47)	1.71 (0.9 –2.5)

HeLa	>3	>3	0.97 (0.58 – 1.16)	2.72 (1.95 – 3.78)
HepG2	>3	>3	1.14 (0.84 – 1.54)	1.4(0.9 – 2.1)
BJ	>3	>3	1.09 (0.28 – 4.2)	1.23 (0.65 – 2.2)

Values represent IC_{50} values with their 95% confidence interval in brackets. Highest concentration of the compounds was 3mM. Where the inhibition did not reach 50% even at this highest concentration, IC_{50} was regarded as higher than 3mM.

Figures:

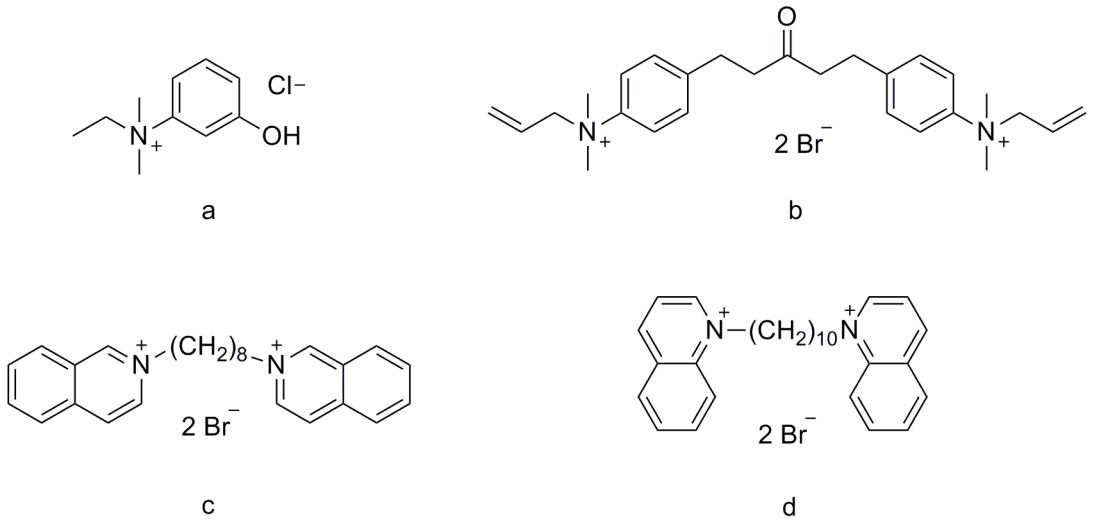

Fig.1: Standard (a, b) and novel (c, d) AChEI

a) Edrophonium (*N*-ethyl-3-hydroxy-*N*,*N*-dimethylbenzenaminium)
b) BW284c51 (1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide)
c) K298 (1,1'-oct-1,8-diyl-bis(quinolinium) dibromide) , d) K524 (1,10-bis(isoquinolinium)-dec-1,10-diyl dibromide)

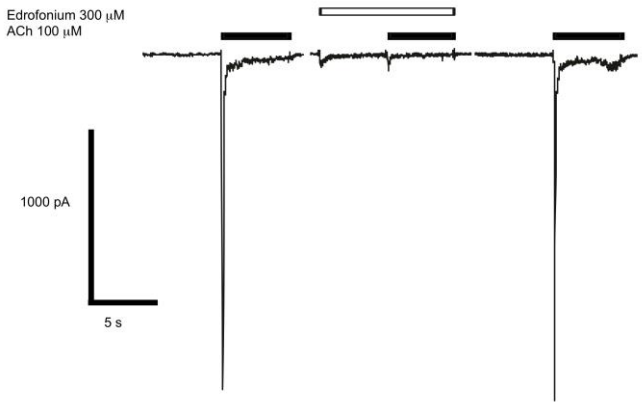

Fig. 2: Edrophonium- induced ion currents. Muscle-type of nicotinic receptor ($\alpha_1\beta_1\gamma\delta$) was stimulated (5s) by ACh (100 μM). Edrophonium (300 μM) was then applied alone (5s) and then together with ACh (5s) to the same cell. Finally, control response was performed.

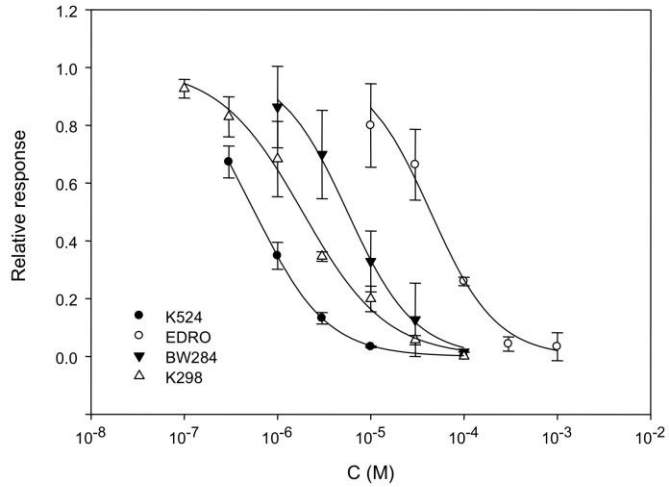

Fig. 3: Antagonistic effect of tested AChEIs

Inhibitory effect of tested AChEIs (K524, Edrophonium, BW283c51 and K298) on ACh- induced currents. Decrease of ACh (100 μM) response amplitudes with increasing concentrations (10^{-7} – 10^{-4} M) of AChEIs. AChEIs were pre-applied 5s before ACh.

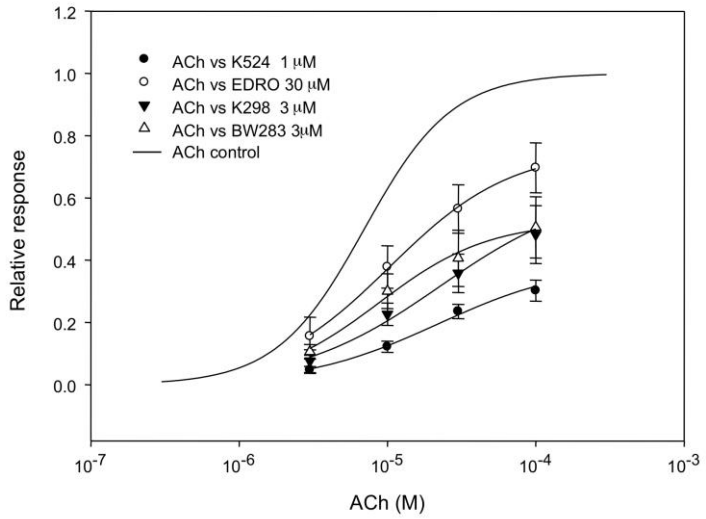

Fig. 4: ACh dose-response curves in the presence of AChEI

Effect of AChEIs (concentration close to IC_{50}) on ACh dose-response curve for ACh was observed after application of ACh alone and than co-applied with individual AChEIs. The relative amplitudes of the responses are normalized to the corresponding maximal response of ACh.