Aus dem Walther-Straub-Institut für Pharmakologie und Toxikologie
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Untersuchung der Affinität und Funktionalität von Bispyridinium-verbindungen an nikotinischen Acetylcholinrezeptoren der Subtypen αβδγ (*Torpedo californica*) und α7 (*Homo sapiens*)

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„Causarum enim cognitio cognitionem eventorum facit!"

Die Kenntnis der Ursachen bewirkt die Erkenntnis der Ergebnisse!

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Für Inge, Nina und Traute

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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München, den 07.07.2019

Karin Veronika Niessen

Inhaltsverzeichnis

1. Abkürzungsverzeichnis

A	Ampere
AChE	Acetylcholinesterase(n)
B_{Max}	Maximale Anzahl an Bindungsstellen (im Äquilibrium)
BuChE	Butyrylcholinesterase(n)
C	Coloumb
Ca	Calcium
CarbE	Carboxylesterase(n)
CHO	Zelllinie aus Ovarien des Chinesischen Zwerghamsters (*Cricetulus griseus*)
CMS	Kongenitales myasthenisches Syndrom (engl. congenital myasthenic syndrome)
CVX	S-[2-(Diisopropylamino)ethyl]-O-butyl-methylphosphonothioat („chinesisches VX"; CV)
CWÜ	Chemiewaffenübereinkommen (Konvention zum Verbot chemischer Waffen)
Cys	Cystein
E605	Parathion (O,O-Diethyl-O-(4-nitrophenyl)phosphorothioat)
f	Femto (10^{-15})
GA	Tabun (O-Ethyl-N,N-dimethylphosphoroamidocyanat)
GB	Sarin (O-Isopropylmethylphosphonofluoridat)
GD	Soman (3,3-Dimethyl-2-butanyl-methylphosphonofluoridat)
GF	Cyclosarin (O-Cyclohexylmethylphosphonofluoridat)
GH_4C_1	Zelllinie aus Tumorzellen der Rattenhypophyse (*Rattus norvergicus*)
GPCR	G-Protein gekoppelte(r) Rezeptor(en) (engl. G-protein coupled receptor)
[^{3}H]	Tritium
HI 6	4-Carbamoyl-1-[({2-[(E)-(hydroxyimino)methyl]-1-pyridiniumyl}methoxy)methyl]-pyridiniumdichlorid („Asoxim")
HLö 7	[1-(2,4-di(Hydroyimino)methyl-1-pyridinium)-1-(4-carboxyaminopyridinium)dimethylether]hydrochlorid
IC_{50}	Mittlere inhibitorische Konzentration
l	Liter
K	Kalium
K_D	Äquilibrium-Dissoziationskonstante („Reporterligand-Affinitätskonstante")
K_i	Äquilibrium-Inhibitionskonstante („Kompetitor-Affinitätskonstante")
LGIC	Liganden gesteuerte(r) Ionenkanal(kanäle) (engl. ion gated ion channel)
M	Mol pro Liter (mol/l)
m	Milli (10^{-3})
µ	Mikro (10^{-6})
mAChR	Muskarinische(r) Acetylcholinrezeptor(en)
MB327	1,1'-(Propan-1,3-diyl)bis(4-*tert*-butylpyridinium)di(iodid)
MB442	1,1'-(Pentan-1,5-diyl)bis(pyridinium)di(iodid)

MB583	1,1’-(Ethan-1,2-diyl)bis(4-*tert*-butylpyridinium)di(iodid)
MB770	1,1‘-(Propan-1,3-diyl)bis(2-phenylpyridinium)di(iodid)
MB779	1,1’-(Decan-1,10-diyl)bis(pyridinium)di(iodid)
MB781	1,1’-(Butan-1,4-diyl)bis(4-*tert*-butylpyridinium)di(iodid)
Mg	Magnesium
n	Nano (10^{-9})
Na	Natrium
nAChR	Nikotinische(r) Acetylcholinrezeptor(en)
NAM	Negative(r) allosterische(r) Modulator(en)
NATO	Organisation des Nordatlantikvertrags (engl. North Atlantic Treaty Organization)
OPC	Phosphororganische Verbindungen (engl. organic phosphoric compounds)
OPCW	Organisation zum Verbot chemischer Waffen (engl. Organisation for the Prohibition of Chemical Warfares)
p	Pico (10^{-12})
PAM	Positive(r) allosterische(r) Modulator(en)
pIC_{50}	Negativer dekadischer Logarithmus des IC_{50}-Werts
pK_i	Negativer dekadischer Logarithmus des K_i-Werts
PNS	Peripheres Nervensystem
PTM0001	1,1’-(Propan-1,3-diyl)bis(3-*tert*-butylpyridinium)di(iodid)
PTM0002	1,1’-(Propan-1,3-diyl)bis(2-*tert*-butylpyridinium)di(trifluormethansulfonat)
S	Siemens
s	Sekunde
SAD-128	1,1’-(Oxydimethylen)bis(4-*tert*-butylpyridinium)di(chlorid)
SAM	Stille(r) allosterische(r) Modulator(en)
SSM	Solid Supported Membrane
SURFE²R	Surface Electronic Event Reader
VC	S-[2-(Diisopropylamino)ethyl]-O-butyl-methylphosphonothioat („chinesisches VX”; CVX)
VR	S-[2-(Diisopropylamino)ethyl]-O-isobutyl-methylphosphonothioat („russisches VX“)
VX	S-[2-(Diisopropylamino)ethyl]-O-ethyl-methylphosphonothioat
ZNS	Zentrales Nervensystem

2. Publikationsliste

2.1. Originalarbeiten

- Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Seeger, T., Thiermann, H., Worek, F. (2011): Interaction of bispyridinium compounds with the orthosteric binding site of human α7 and *Torpedo californica* nicotinic acetylcholine receptors (nAChRs)
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- Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Thiermann, H., Worek, F. (2012): Competition radioligand binding assays for the investigation of bispyridinium compound affinities to the human muscarinic acetylcholine receptor subtype 5 (hM_5)
  Drug Test. Anal. **4**, 292-297

- Niessen, K.V., Seeger, T., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Thiermann, H., Worek, F. (2013): Affinities of bispyridinium non-oxime compounds to [^{3}H]epibatidine binding sites of *Torpedo californica* nicotinic acetylcholine receptors depend on linker length
  Chem. Biol. Interact. **206**, 545-554

- Niessen, K.V., Muschik, S., Langguth, F., Rappenglück, S., Seeger, T., Thiermann, H., Worek, F. (2016): Functional analysis of *Torpedo californica* nicotinic acetylcholine receptors in multiple activation states by SSM-based electrophysiology
  Toxicol. Lett. **247**, 1-10

- Niessen, K.V., Seeger, T., Rappenglück, S., Wein, T., Höfner, G., Wanner, K.T., Thiermann, H., Worek, F. (2018): *In vitro* pharmacological characterization of the bispyridinium non-oxime compound MB327 and its 2- and 3-regioisomers
  Toxicol. Lett. **293**, 190-197

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  Synthesis **49**, 4055-4064

- Scheffel, C., Niessen, K.V., Rappenglück, S., Wanner, K.T., Thiermann, H., Worek, F., Seeger, T., (2018): 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
  Toxicol. Lett. **293**, 157-166

- Scheffel, C., Niessen, K.V., Rappenglück, S., Wanner, K.T., Thiermann, H., Worek, F., Seeger, T., (2018): Counteracting desensitization of human α7-nicotinic acetylcholine receptors with bispyridinium compounds as an approach against organophosphorus poisoning
  Toxicol. Lett. **293**, 149-156

- Seeger, T., Eichhorn, M., Lindner, M., Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, A.C., Thiermann, H., Worek, F. (2012): Restoration of soman-blocked neuromuscular transmission in human and rat muscle by the bispyridinium non-oxime MB327 *in vitro*
  Toxicology **294**, 80-84

- Seeger, T., Niessen, K.V., Langer, P., Gerhardus, J., Worek, F., Friess, H., Bumm, R., Milhaljevic, A.L., Thiermann, H. (2011): Restoration of nerve agent inhibited muscle force production in human intercostal muscle stripes with HI 6
  Toxicol. Lett. **206**, 72-76

- Sichler, S., Höfner, G., Rappenglück, S., Niessen, K.V., Seeger, T., Worek, F., Thiermann, H., Paintner, F.F., Wanner, K.T. (2018): Development of MS Binding Assays targeting the binding site of MB327 at the nicotinic acetylcholine receptor
  Toxicol. Lett. **293**, 172-183

- Wein, T., Höfner, G., Rappenglück, S., Sichler, S., Niessen, K.V., Seeger, T., Worek, F., Thiermann, H., Wanner, K.T. (2018): Searching for putative binding sites of the bispyridinium compound MB327 in the nicotinic acetylcholine receptor
  Toxicol. Lett. **293**, 184-189

3. Einleitung

3.1. Relevanz von Nervenkampfstoffvergiftungen

Die Anschläge mit Sarin während des Syrienkonflikts (Worek et al., 2016b; John et al., 2018), zuletzt im April 2017 (OPCW 2017b), sowie das Attentat auf Kim Jong-nam mit VX (OPCW, 2017a) verdeutlichen, dass Nervenkampfstoffe immer noch eine ernsthafte Bedrohung für die Bevölkerung sind.

Chemisch betrachtet handelt es sich bei Nervenkampfstoffen um Ester und Amide organischer Phosphor- und Phosphonsäuren, die ursprünglich zum Zwecke der Schädlingsbekämpfung eingesetzt wurden. Die militärische Relevanz ergab sich im Jahr 1936, als in den Laboratorien der IG Farben das hochtoxische Tabun (NATO-Bezeichnung „GA“) synthetisiert wurde. Danach erfolgte die Entwicklung weiterer Kampfstoffe, wie etwa Sarin (GB), Soman (GD), Cyclosarin (GF) und in den 1950er Jahren die als „V-Stoffe“ eingruppierten Verbindungen VX, VR („russisches VX“) und VC („chinesisches VX“; auch oft als „CVX“ abgekürzt) (Worek et al., 2016b) (Abb. 1).

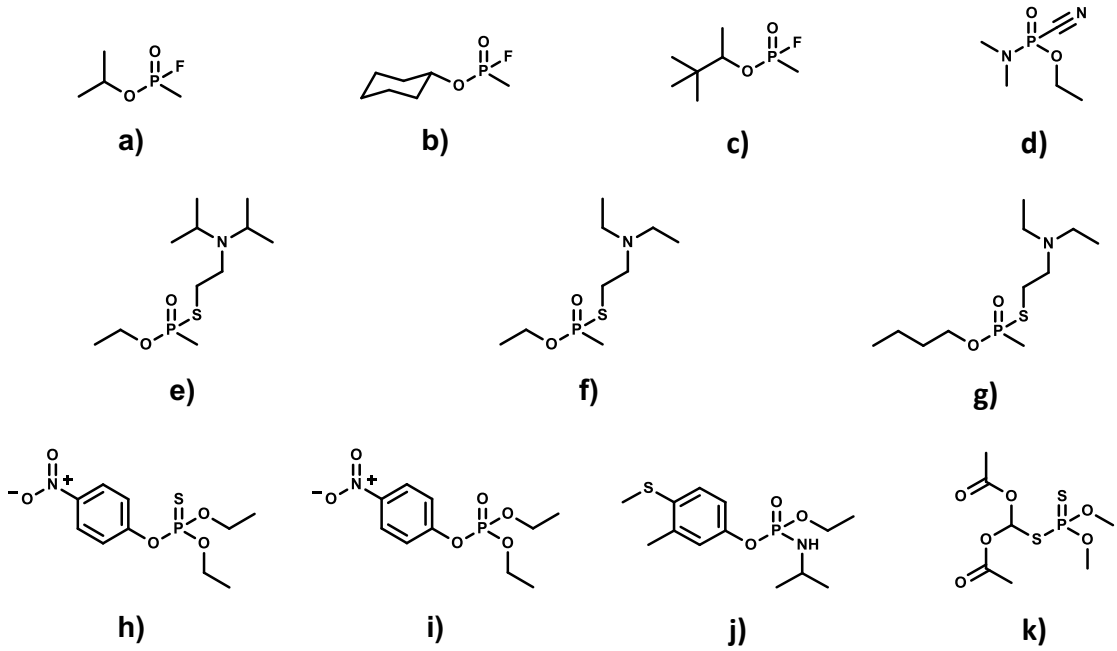


Abb. 1: Nervenkampfstoffe der G-Reihe („G“ für „German“) mit den bekanntesten Vertretern **a)** Sarin, **b)** Cyclosarin, **c)** Soman und **d)** Tabun. Nervenkampfstoffe der V-Reihe („V“ für „Venom“) mit den bekanntesten Vertretern **e)** VX, **f)** russisches VX (VR) und **g)** chinesisches VX (CVX oder VC). Beispiele einiger Pestizide mit **h)** Parathion und dessen aktiven Metabolit **i)** Paraoxon, **j)** Fenamiphos (N-[Ethoxy (3-methyl-4-methylsulfanylphenoxy)phosporyl]propan-2-amin) und **k)** Malathion (Diethyl-2-[(dimethoxyphosphorothioyl)sulfanyl]succinat).

Tausend weitere phosphororganische Verbindungen (OPC) wurden synthetisiert, darunter auch potente Pestizide wie beispielsweise Parathion (O,O-Diethyl-O-(4-nitrophenyl)phosphorothioat; E605), das im Organismus zu Paraoxon (Diethyl-4-nitrophenylphosphat) transformiert wird (Abb. 1) (Kwong, 2002). Für den humanen Organismus sind die pho-

phororganischen Pestizide in der Regel geringer toxisch als Nervenkampfstoffe, verteilen sich aber aufgrund ihrer höheren Lipophilie besser in tiefere Kompartimente (z.B. Fettgewebe), woraus sie kontinuierlich freigesetzt werden können (Thiermann et al., 2007). Im Gegensatz zu den Nervenkampfstoffen fallen die phosphororganischen Pestizide nicht unter dem im Jahr 1997 in Kraft gesetzten Chemiewaffenübereinkommen (Ausführungsgesetz zum Chemiewaffenübereinkommen, 1994). Wenngleich das Chemiewaffenübereinkommen mittlerweile von fast allen Staaten ratifiziert wurde, muss trotzdem aufgrund der schwierig durchzuführenden Kontrollmaßnahmen, der leichten Zugänglichkeit zu Synthesevorschriften und der Existenz restlicher Lagerbestände die Möglichkeit zukünftiger Chemiewaffeneinsätze ernst genommen werden. Insbesondere terroristische Hintergründe gewinnen immer mehr an Bedeutung (Worek et al., 2016b).

3.2. Mechanismus der Toxizität

Die toxische Wirkung von Nervenkampfstoffen beruht auf einer Phosphorylierung bzw. Phosphonylierung der Serin^{203}-Hydroxylgruppe (Ekström et al., 2007) im aktiven Zentrum der Acetylcholinesterase (AChE, E.C. 3.1.1.7), wodurch es zur Inhibition der katalytischen Aktivität kommt (MacPhee-Quigley et al., 1985; Taylor et al., 1995; Marrs, 1993). Eine kovalente Bindung mit OPC gehen auch andere Serinhydrolasen ein, z.B. Butyrylcholinesterase (BuChE, E.C. 3.1.1.8), Carboxylesterase (CarbE, E.C. 3.1.1.1) oder Trypsin (E.C. 3.4.21.4) (Worek et al., 2005), doch insbesondere die Hemmung der AChE ruft rasch eine lebensbedrohliche Situation hervor.

Acetylcholin ist einer der wichtigsten Neurotransmitter im zentralen und peripheren Nervensystem sowie der neuromuskulären Endplatte. Das Enzym AChE katalysiert die Hydrolyse des Neurotransmitters Acetylcholin mit einer beeindruckend hohen Rate (ca. 10.000 Moleküle pro Sekunde!) und reguliert dadurch dessen Konzentration im synaptischen Spalt (Quinn, 1987). Ist die katalytische Aktivität der AChE blockiert, kommt es aufgrund der weiteren Freisetzung zur Akkumulation des endogenen Acetylcholins im synaptischen Spalt (Molenaar et al., 1987). Dadurch wird die Funktion sowohl muskarinischer Acetylcholinrezeptoren (mAChR), die der Klasse der G-Protein gekoppelten Rezeptoren (GPCR) angehören, als auch nikotinischer Acetylcholinrezeptoren (nAChR), bei denen es sich um Ligand-gesteuerte Ionenkanäle handelt, beeinträchtigt. Die dadurch gestörte Reizweiterleitung führt unbehandelt zur cholinergen Krise und letztendlich zum Tod durch einen peripheren und zentralen Atemstillstand (Grob, 1956; Rickett et al., 1986; Marrs, 1993) (Abb. 2).

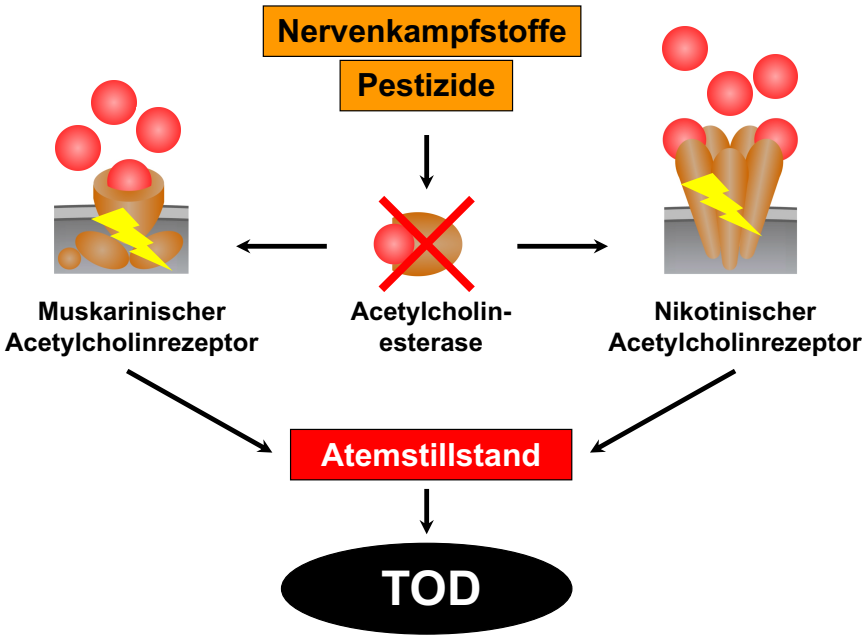


Abb. 2: Vereinfachte Darstellung der Pathophysiologie einer Nervenkampfstoffvergiftung. Die Inhibition der Acetylcholinesterase durch Nervenkampfstoffe oder strukturähnliche Pestizide zieht nach sich, dass der Neurotransmitter Acetylcholin nicht mehr abgebaut wird und im synaptischen Spalt akkumuliert. Dies führt zur Dysfunktion von muskarinischen und nikotinischen Acetylcholinrezeptoren. Unbehandelt führt die dadurch ausgelöste cholinerge Krise zum zentralen und peripheren Atemstillstand und letztendlich zum Tod.

Im humanen Organismus machen sich klinische Symptome bemerkbar, wenn die Aktivität der Erythrozyten-AChE nur noch ca. 20-25% der Normalaktivität beträgt (Thiermann et al., 2009). Das klinische Bild einer akuten Nervenkampfstoffvergiftung wird abhängig von den beteiligten Rezeptoren und deren Lokalisation hauptsächlich durch folgende Symptome geprägt (Grob, 1956; Marrs, 1993):

- peripher-muskarinerg: Überstimulierung von mAChR der exokrinen Drüsen (übermäßiger Tränen- und Speichelfluss, Schweißausbrüche), der glatten Muskulatur (Bronchorrhoe, Bronchokonstriktion, Miosis, abdominale Krämpfe) und des kardialen Systems (Bradykardie),

- peripher-nikotinerg: Übererregung von nAChR des sympathischen Nervensystems (Hypertonie, Tachykardie) und der neuromuskulären Endplatte (Muskelschwäche, Muskelkrämpfe, Paralyse),

- zentralnervös: Überstimulierung von mAChR und nAChR des zentralen Nervensystems (Sprachstörungen, Schwindel, Unruhe, zentrale Atemdepression, epileptische Anfälle).

Die Neusynthese einer funktionalen synaptischen AChE vollzieht sich über 3-5 Tage (Grubić et al., 1981), ist aber für eine Wiederherstellung der Neurotransmission nicht ausreichend, vor allem wenn Nervenkampfstoffe aus anderen Kompartimenten (z.B. subkutanes Fettgewebe) freigesetzt werden und über längere Zeit im Organismus zirkulieren.

Allerdings gibt es biochemische Möglichkeiten, die Aktivität der inhibierten AChE wiederherzustellen. Der Enzym-OPC-Komplex (Abb. 3c) ist nämlich dem Angriff von Nucleophilen zugänglich, wie beispielsweise Oximen (Abb. 3d), so dass aufgrund der Dephosphylierung wieder freies Enzym entstehen kann (Marrs et al., 2006).

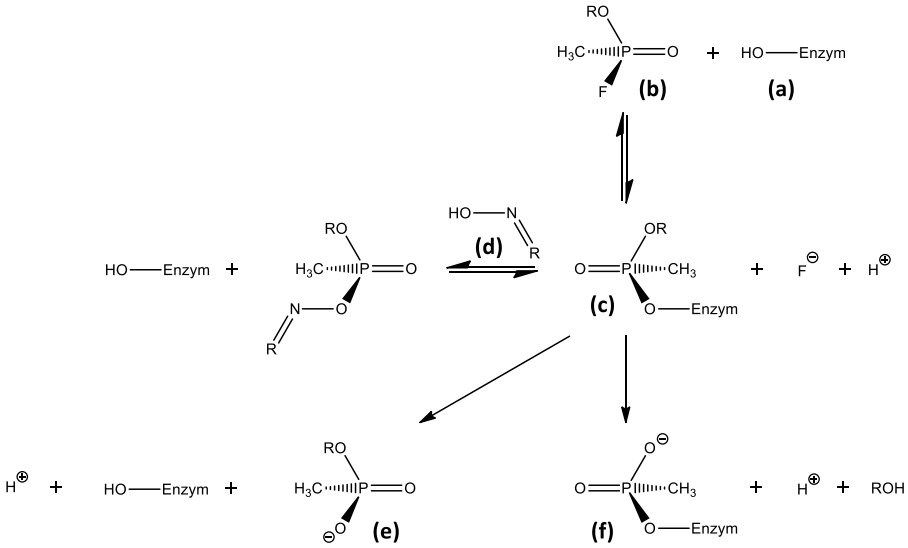


Abb. 3: Reaktion zwischen der AChE **(a)** und Nervenkampfstoff **(b)** und der daraus resultierende Enzym-Gift-Komplex **(c)**. Oxime **(d)** führen zur Reaktivierung der AChE, die in einigen Fällen durch Bildung des Hydrolyseprodukts **(e)** spontan erfolgen kann. Der durch Dealkylierung („Alterung“) gebildete Komplex **(f)** ist einer Reaktivierung durch Oxime nicht mehr zugänglich. Nach (Worek et al., 2004).

Die Freisetzung des Enzyms kann auch durch eine spontane Reaktivierung (Abb. 3e) erfolgen, die in der Regel nur zu einem geringen Anteil erfolgt und in den meisten Fällen alleine nicht ausreicht, um die lebensbedrohliche Situation zu beenden (Worek et al., 2004).

Äußerst problematisch kann sich ein weiterer intramolekularer Prozess, die Dealkylierung („Alterung“) des Enzym-Gift-Komplexes (Abb. 3f) äußern. Insbesondere dann, wenn die Halbwertszeit dieser intramolekularen Alkylabspaltung dermaßen gering ist, bilden sich rasch Spaltungsprodukte und der resultierende AChE-Komplex ist einer Reaktivierung durch Oxime nicht mehr zugänglich. Beispielsweise liegt bei Soman die Halbwertszeit im Minutenbereich (Worek et al., 2004).

In einigen Fällen ist eine chemische Zugänglichkeit zur Reaktivierung generell nicht gegeben. Im Tabun-AChE-Komplex ist aufgrund des Ionenpaars der Aminogruppe die elektronische Dichte des Phosphoratoms erhöht, so dass ein nucleophiler Angriffs durch die Oximgruppe erschwert wird (Worek et al., 2004). Darüber hinaus wurde mittels Kristallstrukturanalyse nachgewiesen, dass durch eine Konformationsänderung im aktiven Zentrum der Zugang für Oxime sterisch gehindert wird (Ekström et al., 2007; Legler et al., 2015).

3.3. Therapie

Aufgrund des raschen Fortschritts des cholinergen Syndroms und der drohenden Lähmung der Atemmuskulatur muss die Behandlung möglichst schnell eingeleitet werden (Newmark, 2004; Eddleston et al., 2008). Die derzeit etablierte Behandlung umfasst Beatmung und den Einsatz von Antidoten (siehe unten), letzteres beim Militär auch als Initialbehandlung im Rahmen der Selbst- und Kameradenhilfe. Zusätzlich werden Antikonvulsiva gegeben, um die epilepsieartigen Krämpfe zu lindern, die durch das überschüssige Acetylcholin ausgelöst bzw. verstärkt wurden (Shih et al., 2007).

3.3.1. Derzeit eingesetzte Antidote

Aktuell werden Vergiftungen mit phosphororganischen Verbindungen mit einer Kombinationstherapie aus Atropin und Oximen (z.B. Obidoxim, Pralidoxim) behandelt (Newmark, 2004; Marrs et al., 2006; Eddleston et al., 2008). Der kompetitive Antagonist Atropin verdrängt das Acetylcholin aus den Bindungsstellen der mAChR, nicht aber nAChR, die für die Muskelfunktion entscheidend sind. Über nAChR vermittelte Effekte werden indirekt behandelt. Oxime reaktivieren die gehemmte AChE und stellen dadurch deren katalytische Aktivität wieder her. Die überschüssige Konzentration an Acetylcholin wird wieder normalisiert, so dass sowohl nAChR als auch mAChR in den „normalen“ Funktionalitätszustand zurückversetzt werden.

Wie bereits erwähnt, dephosphylieren Oxime den Enzym-OPC-Komplex und reaktivieren somit die AChE (Abschnitt 3.2.). Jedoch ist die Effektivität der derzeit zugelassenen Oxime wie Obidoxim (1,3-bis(4-Hydroxyiminomethylpyridinium)-2-oxapropandichlorid) und Pralidoxim (2-Pyridiniumaldoximmethochlorid) bei Vergiftungen mit Soman, Cyclosarin oder Tabun limitiert (Worek et al., 2004). Zwar können einige experimentell verfügbare Oxime, z.B. HI 6 und HLö 7 bei einer Cyclosarin-Vergiftung eine ausreichende Reaktivierung der AChE herbeiführen, jedoch keinen ausreichenden Effekt bei Vergiftungen mit Soman (aufgrund der schnellen Alterung) oder Tabun erzielen. Ein sogenanntes „Breitband-Oxim“ ist nicht verfügbar (Worek und Thiermann, 2013). Zwar könnte die Kombination mehrerer Oxime mit komplementärer und systemischer Wirkung ein sinnvolles Konzept sein (Elsinghorst et al., 2013), dies schließt aber nicht vollständig die immer noch bestehenden therapeutischen Lücken (Worek et al., 2016a).

3.3.2. Alternative Therapieansätze

Aufgrund der noch bestehenden Lücken in der derzeitigen Therapie ist eine Suche nach neuen Behandlungsstrategien unumgänglich.

Denkbar wären der Einsatz von stöchiometrischen und katalytischen Scavengern (z.B. chemisch modifizierte Cyclodextrine) oder Bioscavengern (z.B. genetisch veränderte AChE, BuChE oder Paraoxonase), die vor allem zur Vorbehandlung eingesetzt werden könnten (Elsinghorst et al., 2013; Masson und Nachon, 2017). Eine Vorbehandlung mit Carbamaten (z.B. Pyridostigmin, Physostigmin) führt zu einer reversiblen Carbamoylie-

rung der AChE und verhindert somit den Angriff von OPC. Allerdings ist diese Vorbehandlung nicht unumstritten (Masson und Nachon, 2017).

Ein interessanter Therapieansatz wäre die direkte Interaktion von Wirkstoffen an den nikotinischen Acetylcholinrezeptoren, insbesondere an Muskeltyp-nAChR, so dass eine ausreichend hohe Rezeptorfunktion wieder vorhanden ist (Sheridan et al., 2005; Turner et al., 2011). Hierbei müsste die Rezeptorpopulation, in denen sich der Rezeptor im geschlossenen, aber aktivierbaren Zustand befindet, erhöht werden oder der an sich metastabile, aktive Zustand „eingefroren“ werden (siehe auch Abschnitt 3.4.2.).

3.4. Nikotinische Acetylcholinrezeptoren

Bei den nAChR handelt es sich um Ligand gesteuerte Ionenkanäle (LGIC), die analog γ-Aminobuttersäure Subtyp A ($GABA_A$)-, Serotonin Subtyp 3 ($5\text{-}HT_3$)- und Glycin (Gly)-Rezeptoren der Familie der sogenannten Cys-Loop-Rezeptoren angehören (Lester et al., 2004; Sine und Engel, 2006). nAChR sind im Gehirn, in sympathischen und parasympathischen Ganglien, im Skelettmuskel sowie in Epithelial- und Immunzellen weit verbreitet (Gotti und Clementi, 2004; Kalamida et al., 2007; Wu und Lukas, 2011). Sie sind sowohl an der prä- als auch an der postsynaptischen Membran vorhanden (Albuquerque et al., 2009). nAChR sind nicht nur in physiologischen, sondern auch in zahlreichen pathophysiologischen Prozessen im ZNS (z.B. Alzheimer, Parkinson, Schizophrenie, Depression) und im PNS (z.B. kongenitales myasthenisches Syndrom (CMS), Myasthenia gravis) involviert (Gotti und Clementi, 2004; Taly et al., 2009; Sine, 2012; Melzer et al., 2016).

3.4.1. Struktur

Der nAChR besteht aus insgesamt 5 Untereinheiten, die mit den griechischen Buchstaben α, β, γ, δ und ε bezeichnet werden. Es sind derzeit 17 verschiedene Untereinheiten bekannt, deren mannigfaltige Kombination eine Vielzahl von Pentameren ergibt (Albuquerque et al., 2009; Kalamida et al., 2007; Le Novère et al., 2002). Sind die Untereinheiten der Pentamere identisch, wie beispielsweise beim α7-nAChR, werden diese Rezeptoren als monomer klassifiziert. Der sogenannte Muskeltyp-nAChR ist dagegen heteromer; er besteht aus vier verschiedenen Untereinheiten, wovon die α-Untereinheit doppelt vorkommt. Vom Muskeltyp-nAChR, der bei Säugetieren ausschließlich im Skelettmuskel lokalisiert ist, werden die fötale Form $(\alpha1)_2\beta1\delta\gamma$ (oft vereinfacht als $\alpha1\beta1\delta\gamma$, d.h. ohne Stöchiometrie bezeichnet) und die adulte Form $(\alpha1)_2\beta1\delta\varepsilon$ (vereinfachte Bezeichnung $\alpha1\beta1\delta\varepsilon$) unterschieden. Die im elektrischen Organ verschiedener Zitterrochen (*Torpedo*)- und Zitteraal (*Electrophorus*)-Spezies vorhandenen $\alpha\beta\delta\gamma$-nAChR sind ebenfalls dem Muskeltyp zuzuordnen (Millar und Gotti, 2009; Kalamida et al., 2007).

Der Subtyp α7 wird als neuronaler Rezeptorsubtyp beschrieben, scheint aber ubiquitär im Organismus vorhanden zu sein, z.B. auf der Oberfläche von Makrophagen (Kalamida et al., 2007). Insbesondere in pathophysiologischen Situationen, die mit einer Denervierung einhergehen, aber auch nach längerer Immobilisation werden im Skelettmuskel fötale

($\alpha 1\beta 1\delta\gamma$) und α7-nAChR hochexprimiert, die in der Neurotransmission involviert sind und daher als Backup-System fungieren könnten (Khan et al., 2014; Lee et al., 2014; Martyn und Richtsfeld, 2006). Möglicherweise muss auch bei subletalen Nervenkampfstoffvergiftungen mit diesen Prozessen gerechnet werden. Daher rückt nicht nur der Muskeltyp-nAChR, sondern auch der α7-nAChR in den Fokus für die Entwicklung von Antidoten für die Behandlung von Nervenkampfstoffvergiftungen; insbesondere auch dann, wenn es um die Subtyp-Selektivität geht.

Die 5 Untereinheiten sind nicht kovalent miteinander gebunden und so assoziiert, dass sie in der Mitte eine wassergefüllte Pore bilden. Dieser in der Membran integrale Verbund ermöglicht die Passage von Kationen und ist unterteilt in eine extrazelluläre Domäne, Transmembran-Domäne und intrazelluläre Domäne (Unwin, 2005; Unwin, 2013) (Abb. 4).

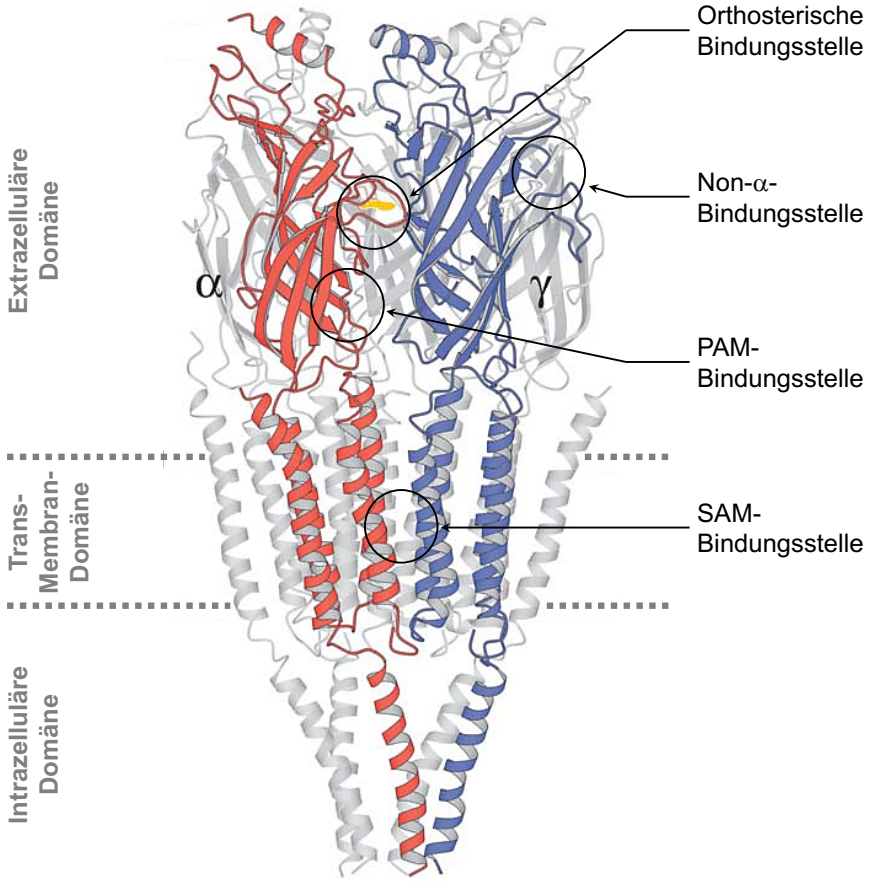


Abb. 4: Schematische Darstellung des nikotinischen Acetylcholinrezeptors (Unwin, 2005) mit einigen der bislang bekannten Bindungsstellen. Alle Bindungsstellen, die sich von der orthosterischen Bindungsstelle unterscheiden, werden als allosterische Bindungsstellen bezeichnet wie beispielsweise diejenige für positiv allosterische Modulatoren (PAM). Synonym für „stille allosterische Modulatoren" (SAM) ist auch „Desensitizer" (Abbildung modifiziert nach (Unwin, 2005), mit Genehmigung des Verlags).

An den extrazellulär lokalisierten Bindungsstellen für den endogenen Liganden Acetylcholin, die als orthosterische Bindungsstellen bezeichnet werden, binden weitere Agonisten (z.B. Epibatidin, Cytisin), aber auch Antagonisten (z.B. α-Bungarotoxin, Conotoxine) (Arias,

2000). Alle anderen Bindungsstellen werden als allosterische Bindungsstellen bezeichnet, wovon die Mehrzahl ebenfalls in der extrazellulären Domäne lokalisiert ist (Arias, 2010) (Abb. 4). Von Bindung und Art des Liganden hängt es ab, welche Funktionalitätszustände des nAChR stabilisiert werden.

3.4.2. Rezeptorfunktion und Aktivierungszustände

Wenn kein Ligand oder ein Antagonist an der orthosterischen Bindungsstelle des nAChR bindet, wird der Ruhezustand stabilisiert. In diesem geschlossenen Zustand sind die 5 Untereinheiten verdrillt, so dass die Passage von Kationen unterbunden wird. Wenn Acetylcholin (oder ein anderer Agonist) die orthosterischen Bindungsstellen belegt, öffnet sich der nAChR durch Rotation der Untereinheiten und leitet durch seine nun erweiterte Pore Na^+- und Ca^{2+}-Ionen nach innen und K^+-Ionen nach außen (Miyazawa et al., 2003; Unwin und Fujiyoshi, 2012; Wang et al., 2008). Der Na^+-Ioneneinstrom hat die Depolarisation der postsynaptischen Membran zur Folge, wodurch weitere Signalkaskaden in Gang gesetzt werden; im neuromuskulären System kommt es letztendlich zur Muskelkontraktion (Martyn et al., 2009).

In hohen Konzentrationen bindet Acetylcholin an zusätzlichen Bindungsstellen mit niedrigerer Affinität als an den orthosterischen Bindungsstellen (Arias, 1996). Dies hat zur Folge, dass der nAChR in einen geschlossenen, den sogenannten desensitisierten Zustand übergeht (Auerbach und Akk, 1998) (Abb. 5).

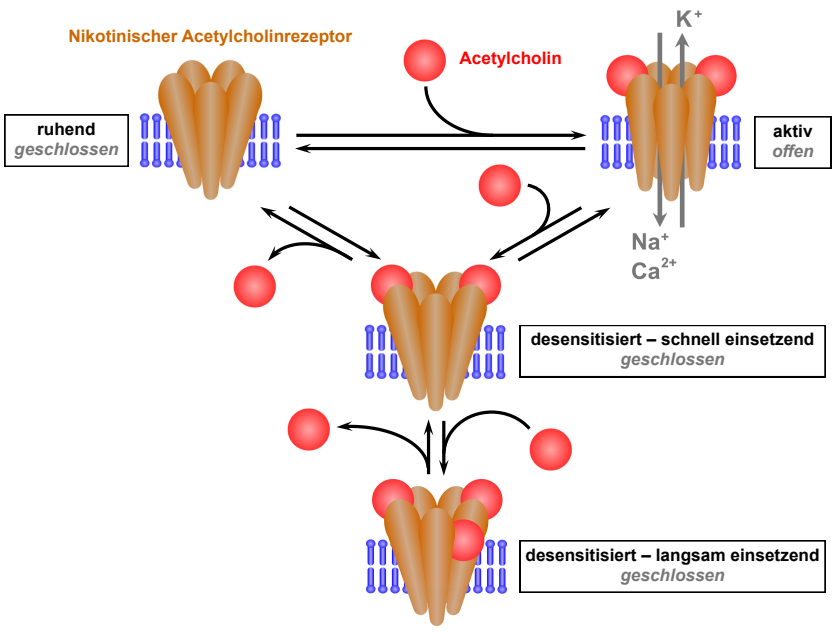


Abb. 5: Vereinfachte Darstellung der verschiedenen Zustände des nAChR. Alle Zustände stellen Wahrscheinlichkeiten dar. Die Zustände „aktiv“ und „desensitisiert – schnell einsetzend“ sind metastabil. Bei einer normalen Neurotransmission ist der „ruhend“-Zustand am wahrscheinlichsten, bei pathologischen Zuständen, die mit einer Neurotransmitteranhäufung einher gehen (z.B. Nervenkampfstoffvergiftung) ist der „desensitisiert – langsam einsetzend“-Zustand am wahrscheinlichsten.

Die Übergänge zwischen den einzelnen Aktivierungsstadien sind fließend und beinhalten zahlreiche Intermediärzustände. Welches Desensitisierungsstadium eintritt, hängt von der Acetylcholin-Konzentration und insbesondere von der Expositionsdauer des Acetylcholins auf die Rezeptoren ab. Chronische Überflutung mit hohen Acetylcholin-Konzentrationen führt zu einer langsam einsetzenden Desensitisierung (vereinzelt auch als „tiefes“ Stadium bezeichnet), deren Aufhebung entsprechend lang dauert (Reitstetter et al., 1999). Ist das Ausmaß an Agonist-Überschuss geringer, wird eine schnell einsetzende Desensitisierung („flaches“ Stadium) hervorgerufen (Reitstetter et al., 1999). Nach anschließender Entfernung des Agonisten sind die nAChR binnen wenigen Minuten wieder aktivierbar (Auerbach und Akk, 1998; Boyd, 1987). Die Kinetik der Desensitisierung und deren Aufhebung hängen nicht nur von der Konzentration und Affinität des Agonisten, sondern auch vom jeweiligen nAChR-Subtyp ab (Papke, 2014). Beispielsweise desensitisieren α7 nAChR innerhalb weniger Millisekunden, während bei den Muskeltyp-nAChR eine Desensitisierung erst im Sekundenbereich eintritt (Giniatullin et al., 2005). Eine schnell einsetzende, vorübergehende Desensitisierung ist womöglich als physiologische Schutzfunktion anzusehen (Giniatullin et al., 2005; Quick und Lester, 2002).

Der Zustand „desensitisiert – schnell einsetzend“ ist also ebenso wie der offene („aktiv“) Zustand metastabil. Folglich ist bei einer normalen Neurotransmission der geschlossene, aber aktivierbare („ruhend“) Zustand am wahrscheinlichsten. Bei pathologischen Situationen, die mit einer zu hohen Neurotransmitterkonzentration im synaptischen Spalt einhergehen, ist der geschlossene, aber nicht aktivierbare („desensitisiert – langsam einsetzend“) Zustand relevant (Karlin, 2002). Die Desensitisierung der nAChR in der neuromuskulären Endplatte führt zu einem Ausfall der neuromuskulären Transmission (Maselli und Leung, 1993), wobei auch weitere Mechanismen wie Phosphorylierung im Bereich der intrazellulären Domäne und Internalisierung diskutiert werden (Stokes et al., 2015; Kumari et al., 2008).

Der genaue Mechanismus der Desensitisierung und wie diese aufgehoben werden kann, ist noch nicht im Detail aufgeklärt. Einer Theorie zufolge wird durch eine stetige Bindung des Agonisten die Energiebarriere zur Desensitisierung überwunden (Papke, 2014). Die Kopplung zwischen der Bindungs- und Durchleitungsregion ist dann aufgrund einer Konformationsänderung gestört (Zhang et al., 2011; Yamodo et al., 2010), d.h. die normalerweise konzertierte Rotation der Untereinheiten durchläuft nicht mehr alle Domänen des Rezeptors, so dass die Pore trotz Bindung des Agonisten geschlossen bleibt. Eine weitere Hypothese bestätigt, dass eine Konformationsänderung für die Desensitisierung ursächlich ist. Die zur α-Untereinheit gehörige und in der Transmembran-Region lokalisierte M4-Domäne ist nicht an die extrazelluläre Cys-Schleife angedockt, so dass die extrazelluläre und die transmembranäre Domäne des nAChR entkoppelt sind (daCosta et al., 2013).

3.4.3. Allosterische Modulatoren

Mit der Einwirkung auf die verschiedenen Energiebarrieren zwischen den geschlossenen, offenen und desensitisierten Zuständen wird auch die Wirkungsweise von allosterischen

Modulatoren erklärt. Es hat gar den Anschein, dass nicht nur die Desensitisierung, sondern auch die allosterische Modulation ein von der Natur vorgesehener Mechanismus ist, um die Funktion der nikotinischen Acetylcholinrezeptoren zu kontrollieren (Pereira et al., 2002; Quick und Lester, 2002). Sowohl negativ allosterische Modulatoren (NAM) als auch positiv allosterische Modulatoren (PAM) haben gemeinsam, dass sie an anderen Stellen binden als Agonisten und ihre Wirkung in Gegenwart von Agonisten ausüben (Arias, 2010). Allosterische Bindungsstellen werden aber auch von Substanzen belegt, die den densitisierten Zustand stabilisieren, beispielsweise Agonisten im Überschuss. Die Stabilisierung desensitisierter Zustände ist typisch für sogenannte stille allosterische Modulatoren (SAM) (Papke et al., 2015). Definitionsgemäß beeinflussen SAM nicht die Wirkung des orthosterischen Liganden, sondern verhindern die Ausübung jeglicher Modulation aufgrund der Verdrängung von PAM (oder NAM) aus ihren allosterischen Bindungsstellen (Christopoulos et al., 2014; Chatzidaki und Millar, 2015). Bezüglich der intrinsischen Aktivität, die auf den nAChR ausgeübt wird, haben Antagonisierung und Desensitisierung gemeinsam, dass es abhängig vom Ausmaß zu keiner Durchleitung von Kationen kommt.

Für positive allosterische Modulatoren sind zwei Mechanismen denkbar, die aber in beiden Fällen die Erhöhung der Population an offenen Zuständen zur Folge haben (Chatzidaki und Millar, 2015; Williams et al., 2011; Papke, 2014):

- Die Energiebarriere zwischen dem geschlossenen und dem offenen Zustand wird herabgesetzt,
- Die Energiebarriere zwischen dem offenen und dem desensitisierten Zustand wird heraufgesetzt.

Gemäß ihrer Wirkungsweise werden positiv allosterische Modulatoren (PAM) in zwei Klassen eingeteilt: Typ I PAM und Typ II PAM (Chatzidaki und Millar, 2015). Beide Typen haben gemeinsam, dass sie die Affinität von Agonisten erhöhen und den Durchstrom der Kationen verstärken, d.h. die Population der geschlossenen verschiebt sich zugunsten der aktiven Zustände. Doch nur Typ II PAM verzögern den Eintritt der Desensitisierung und sind darüber hinaus in der Lage, bereits desensitisierte Rezeptoren in den offenen Zustand zu versetzen (Chatzidaki und Millar, 2015).

Von Oximen mit Bispyridinium-Struktur wurde vermutet, dass sie nicht nur die AChE reaktivieren, sondern auch direkte Effekte am nAChR ausüben könnten. Sowohl *in vitro* (Wolthuis et al., 1981; French et al., 1983) als auch *in vivo* (Hamilton und Lundy, 1989; van Helden et al., 1991) stellte sich heraus, dass diese Substanzen selbst dann einen positiven pharmakologischen Effekt zeigten, wenn die AChE einer Reaktivierung nicht zugänglich war, z.B. bei „gealterter“ AChE nach einer Somanvergiftung. Auch SAD-128, ein Strukturabkömmling von Obidoxim, jedoch ohne Oximgruppen, wirkte zusammen mit Atropin als Antidot bei Mäusen und Meerschweinchen nach Vergiftungen mit Soman und auch anderen Nervenkampfstoffen (Schoene und Oldiges, 1973; Inns und Leadbeater, 1983). Es wurde vermutet, dass die Effekte möglicherweise mit der Bispyridinium-Struktur zusammenhingen und sich antagonistisch auf die nAChR auswirkten (Alkondon und Albu-

querque, 1989). Intensive Forschung des Instituts „Defence Science and Technology Laboratory“ (Dstl) Porton Down, Salisbury, England, identifizierte mit MB327 eine Bispyridiniumverbindung, die ebenfalls keine Oximgruppe aufweist und sowohl *in vivo* als auch *in vitro* positive pharmakologische Effekte bei der Behandlung von Somanvergiftungen zeigte, ohne dass die gehemmte AChE wieder reaktiviert wurde (Turner et al. 2011; Timperley et al. 2012). Allerdings war noch nicht vollständig bewiesen, ob die Effekte wirklich über nAChR vermittelt waren und wenn ja, in welcher Weise die Bispyridiniumverbindungen mit den nAChR interagieren.

Auch wenn MB327 insbesondere bei Somanvergiftungen einen therapeutischen Benefit zeigte, ist es als potentielles Arzneimittel nicht geeignet, da relativ hohe Mengen eingesetzt werden müssten. Kürzlich stellte sich heraus, dass bei hohen Konzentrationen toxische Eigenschaften zu beobachten waren (Price et al., 2016), da offensichtlich auch andere Zielstrukturen (z.B. mAChR) adressiert werden. Zukünftiges Ziel ist daher die Entwicklung potenterer und Subtyp-selektiver Liganden (Jensen et al., 2005; Changeux und Taly, 2008), wofür aussagekräftige Screening-Methoden zur Verfügung stehen müssen.

3.5. Fragestellungen und Ziele

Wie vorhin erwähnt, zeigten einzelne Bispyridiniumverbindungen *in vitro* und *in vivo* positive pharmakologische Effekte, aber es fehlte noch ein zusammenhängendes Bild über die genaue Wirkungsweise, das für die Entwicklung potenterer Wirkstoffe unabdingbar ist. Insbesondere folgende Fragestellungen waren noch unbeantwortet:

- Interagieren die Bispyridiniumverbindungen direkt mit den nAChR?
- Mit welcher Bindungsstelle interagieren sie und lassen sich hierbei Struktur-Wirkungsbeziehungen erkennen?
- Binden Bispyridiniumverbindungen auch an mAChR?
- Welche intrinsische Aktivität üben die Bispyridiniumverbindungen am nAChR aus?
- Lassen sich die Effekte der Bispyridiniumverbindungen, die in verschiedenen Modellen (Muskeltyp-nAChR, Rattendiaphragma) festgestellt wurden, zu einem Gesamtbild zusammenfügen?

Um die Wirkweise möglicher Antidote bei OPC-Vergiftungen aufzuklären und um bessere Wirkstoff-Kandidaten zu entwickeln, ist ein iterativer Prozess aus zielgerichteter Synthese, Affinitäts- und Funktionalitätsuntersuchungen am Rezeptor und letztendlich Experimenten am Gewebe zielführend (Abb. 6).

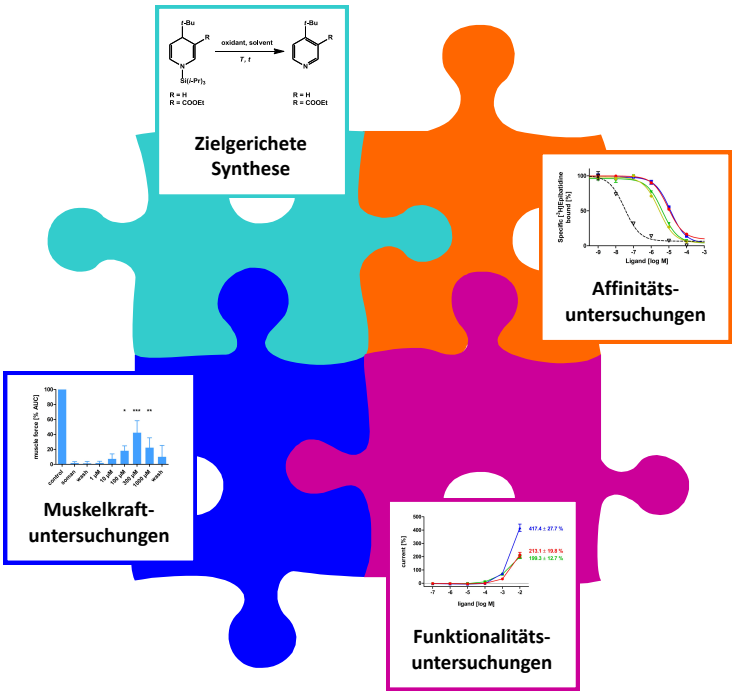
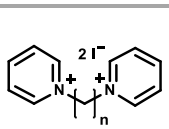
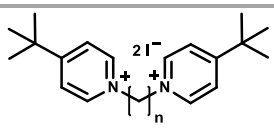


Abb. 6: Das rationale Wirkstoffdesign beruht auf die Verzahnung von der Synthese (inklusive Molecular Modeling) mit verschiedenen Screeningmethoden. Die Muskelkraftexperimente beleuchten den Vorgang der neuromuskulären Transmission, die Bausteine „Affinitätsuntersuchungen“ und „Funktionalitätsuntersuchungen“ befassen sich mit der direkten Interaktion an den Rezeptoren.

Die Substanzbibliotheken wurden extern bezogen: einerseits „MB“-Substanzen von Dstl Porton Down, Salisbury, England und andererseits „PTM“-Substanzen vom Arbeitskreis Prof. Dr. Wanner, Zentrum für Pharmaforschung, Ludwig-Maximimilians-Universität (LMU) München.

Gegenstand dieser Dissertation war das Screening auf rezeptoraktive Substanzen mit der Beschränkung auf symmetrisch aufgebaute Bispyridiniumverbindungen. Für die Identifikation möglicher Struktur-Wirkungsbeziehungen wurden einerseits die Länge der Alkylbrücke zwischen den beiden Pyridiniumringen und andererseits das Substitutionsmuster dieser beiden Heterozyklen betrachtet. Die Länge des Alkyl-Spacers umspannte die Methylengruppe (C1) bis hin zum *n*-Decyl-Linker (C10) von sowohl unsubstituierten als auch 4-*tert*-Butyl-subsituierten Bispyridiniumverbindungen (Tab. 1).

Sämtliche Verbindungen waren symmetrisch, d.h. sie wiesen eine Symmetrieachse auf.

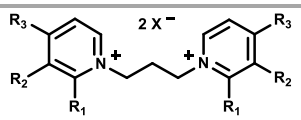
n		
1	MB775	MB780
2	MB520	MB583
3	MB408	MB327
4	MB444	MB781
5	MB442	MB782
6	MB776	MB783
7	MB777	MB784
8	MB505	MB785
9	MB778	MB582
10	MB779	MB786

Tab. 1: Die getesteten Bispyridiniumverbindungen mit Variationen in der Alkyl-Brücke. Sowohl bei den unsubstituierten als auch bei den 4-*tert*-Butyl-substituierten Strukturanaloga variierte die Länge vom Methyl- (n=1) bis hin zum *n*-Decyl-Linker (n=10).

Ausgehend von der Leitsubstanz MB327, deren beiden *tert*-Butylpyridinium-Ringe mit einer *n*-Propyl (C3)-Kette miteinander verbunden sind, wurden Strukturanaloga mit verschiedenen Substitutionsmustern betrachtet. Sämtliche Substitutionen der beiden Heterozyklen waren zueinander spiegelbildlich, d.h. auch diese Verbindungen besaßen eine Symmetrieachse (Tab. 2).

Mit eingeschlossen waren Methyl-Regioisomere (MB420, MB419, MB414), Ethyl-Regioisomere (MB455, MB421, MB454) und *tert*-Butyl-Regioisomere (PTM0002, PTM0001, MB327), d.h. mit Substitutionen entweder in der 2-Position (R_1), 3-Position (R_2) oder 4-Position (R_3) beider Pyridinium-Ringe (Tab. 2).

Die beiden Strukturanaloga PTM0042 und PTM0045 (Tab. 2) wurden eingesetzt, um Effekte auszuschließen, die eventuell durch unterschiedliche Gegenionen verursacht werden und vor allem bei elektrophysiologischen Methoden eine Rolle spielen können. Beispielsweise könnten bestimmte Ionen die Rezeptor-umgebende Membran durch elektrostatische Wechselwirkungen destabilisieren, so dass möglicherweise die zu messenden Kationen direkt die Membran durchströmen und somit rezeptorvermittelte Effekte vortäuschen.

				
Modifikation	**R_1**	**R_2**	**R_3**	**X^-**
MB456	—	Bromid-	—	Bromid
MB424	—	—	Trifluormethyl-	Iodid
MB770	Phenyl-	—	—	Iodid
MB420	Methyl-	—	—	Iodid
MB419	—	Methyl-	—	Iodid
MB414	—	—	Methyl-	Iodid
MB455	Ethyl-	—	—	Iodid
MB421	—	Ethyl-	—	Iodid
MB454	—	—	Ethyl-	Iodid
PTM0002	*tert*-Butyl-	—	—	Triflat
PTM0042	*tert*-Butyl-	—	—	Iodid
PTM0001	—	*tert*-Butyl-	—	Iodid
MB327	—	—	*tert*-Butyl-	Iodid
PTM0045	—	—	*tert*-Butyl-	Triflat

Tab. 2: Die getesteten Propan-Bispyridiniumverbindungen mit verschiedenen Substitutionsmustern. Die beiden Verbindungen PTM0042 und PTM0045 wurden nur bei der SSM-basierten Elektrophysiologie eingesetzt, um einen möglichen Einfluss verschiedener Gegenionen (X^-) zu untersuchen. „Triflat“ ist die gängige Kurzbezeichnung für „Trifluormethansulfonat“.

Zu Beginn dieser Dissertation waren weder Affinitäts- noch Funktionalitätsmethoden etabliert. Plasmamembranpräparationen, die humane mAChR enthalten, waren kommerziell verfügbar, nicht aber solche mit nAChR. Für die Bearbeitung der Fragestellungen betreffend der nAChR musste die Methodik von der Kultivierung rekombinanter Systeme bis hin zu Plasmamembranpräparation aufgebaut werden. Sowohl für mAChR als auch für nAChR wurden Affinitätsassays auf Grundlage der Filtrationstechnik und unter Verwendung von Tritium markierten Liganden entwickelt, die Subtyp-unselektiv, d.h. für alle Rezeptorsubtypen geeignet waren. Für Affinitätsassays mit nAChR standen der hochaffine Agonist [^{3}H]Epibatidin und für die mAChR-Affinitätsassays der hochaffine Antagonist [^{3}H]N-Methylscopolamin zur Verfügung. Im Gegensatz zum humanen α7 nAChR war für den adulten humanen Muskeltyp ($\alpha 1 \beta 1 \delta \varepsilon$) kein geeignetes Expressionssystem verfügbar, so dass auf den strukturell sehr ähnlichen Muskeltyp-nAChR ($\alpha\beta\delta\gamma$) des Zitterrochens zurückgegriffen wurde (siehe Abschnitt 3.5.1). Die aus dem elektrischen Organ gewonnenen, nAChR enthaltenden Proteinsuspensionen waren einer konventionellen Funktionalitätsuntersuchung unter Einsatz der Patch Clamp-Technik nicht zugänglich, da hierfür ganze Zellen erforderlich waren. Speziell hierfür wurde eine Bilayer-basierte elektrophysiologische Methode entwickelt, die für alle nAChR-Subtypen in Proteinsuspensionen geeignet ist und direkt den nAChR in seinem desensitisierten, pathophysiologischen Zustand untersucht.

Muskelkraftmessungen am Rattendiaphragma waren bereits etabliert, unter anderem ein sogenanntes Multiorganbad, das bis zu 12 simultane Messungen erlaubt (Seeger et al., 2007). Für die Muskelkraftmessungen werden hauptsächlich Diaphragma-Hemisphären aus Ratten eingesetzt, die eine vergleichbare Pharmakologie mit humanen Interkostalmuskeln zeigten (Seeger et al., 2012). Zwar wären Präparate aus humaner Atemmuskulatur ideal, sind aber aufgrund verfeinerter Operationstechniken („Schlüsselloch-Operationen“) nicht mehr in ausreichend hohen Mengen verfügbar.

Die in dieser Dissertation verwendeten Modelle auf Rezeptor- und Gewebe-Ebene sollen dann letztendlich zeigen, ob die pharmakologischen Wirkungen der getesteten Substanzen ein zusammenhängendes Bild ergeben und so die Grundlage für die Entwicklung neuartiger potentieller Antidote für die Behandlung von Nervenkampfstoffen schaffen.

3.5.1. Affinität an der orthosterischen nAChR-Bindungsstelle

Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Seeger, T., Thiermann, H., Worek, F. (2011): Interaction of bispyridinium compounds with the orthosteric binding site of human α7 and *Torpedo californica* nicotinic acetylcholine receptors (nAChRs)
Toxicol. Lett. **206**, 100-104

Die erste Publikation behandelt die Interaktion von Bispyridiniumverbindungen an der orthosterischen Bindungsstelle der nAChR-Subtypen *Homo sapiens* α7 und *Torpedo californica* αβδγ.

Für die Affinitätsuntersuchungen wurden Plasmamembranpräparationen eingesetzt. Einerseits wurden diese aus GH_4C_1-Zellen, die den humanen α7-nAChR stabil überexprimieren, und andererseits aus dem elektrischen Organ des kalifornischen Zitterrochens (*Torpedo californica*) gewonnen, die eine relativ hohe Homologie zu humanen Muskeltyp-nAChR aufweisen (Navedo et al., 2004). Eine rekombinante Herstellung des humanen adulten Muskeltyps (α1β1δε-nAChR) ist bislang nur in transienten, jedoch nicht in stabilen Expressionssystemen gelungen (Millar, 2009) gelungen. Für die Herstellung der Membranpräparationen sind aber hohe Zellmassen erforderlich, deren Produktion in transienten Expressionssystemen viel zu unwirtschaftlich ist. Die Expression eines funktionalen Rezeptors mit der richtigen Stöchiometrie ist ein äußerst komplizierter und immer noch nicht vollends aufgeklärter Prozess (Millar und Harkness, 2008; Valkova et al., 2011). Für die Entwicklung eines stabilen Expressionssystems für α1β1δε-nAChR müssen noch genauere Kenntnisse insbesondere in translationalen Mechanismen gewonnen werden.

Die Affinitätsassays wurden auf Grundlage eines Filtrationsassays im 96 well-Format und unter Einsatz von Festszintillatoren entwickelt. Als Reporterligand wurde das mit Tritium markierte [^{3}H]Epibatidin eingesetzt (Houghtling et al., 1995), wobei es sich um einen Agonisten handelt, der mit hoher Affinität an die orthosterische Bindungsstelle bindet.

Sättigungsassays zeigten den enormen Unterschied zwischen den B_{Max}-Werten (maximale Anzahl an Bindungsstellen) der beiden Plasmamembranpräparationen. Bei den sich anschließenden Kompetitionsexperimenten wurde das Verhältnis zwischen Bindungsstellen und [^{3}H]Epibatidin-Konzentration standardisiert.

Einige der ausgewählten Bispyridiniumverbindungen inhibierten die [^{3}H]Epibatidinbindung bei beiden nAChR-Subtypen, wobei die Affinitäten im mikromolaren Bereich lagen. Sowohl an humanen α7-nAChR als auch an *Torpedo*-nAChR interagierte mit den [^{3}H]Epibatidin-Bindungsstellen von allen untersuchten Bispyridiniumverbindungen das Phenyl-substituierte Derivat MB770 mit der stärksten Affinität. Allerdings stellte sich später heraus, dass MB770 im Diaphragmen-Test keinerlei muskelaktivierende Wirksamkeit zeigte. Das *in vivo* und *in vitro* wirksamste MB327 führte bei humanen α7-nAChR und *Torpedo*-nAChR zunächst zu einer Affinitätszunahme und bei höheren Konzentrationen zu einer Inhibition der [^{3}H]Epibatidinbindung.

Es gab somit erste Hinweise, dass ausgewählte Bispyridiniumverbindungen mit den nAChR interagieren. Insbesondere die Affinitätszunahme des orthosterischen Liganden [^{3}H]Epibatidin lässt auf allosterische Effekte schließen.

Mittlerweile konnte mit Bindungsassays auf Basis der Massenspektrometrie („MS Bindungsassays“) gezeigt werden, dass sich die MB327-Bindung absättigen läßt, dass also tatsächlich konkrete Bindungsstellen durch MB327 besetzt werden (Sichler et al., 2018).

3.5.2. Affinitäten am humanen mAChR

Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Thiermann, H., Worek, F. (2012): Competition radioligand binding assays for the investigation of bispyridinium compound affinities to the human muscarinic acetylcholine receptor subtype 5 (hM_5)
Drug Test. Anal. **4**, 292-297

Neben den Affinitätsassays mit nikotinischen Acetylcholinrezeptoren galt es nun, die Affinitäten an muskarinischen Acetylcholinrezeptoren zu untersuchen. In der zweiten Publikation wird die Entwicklung von Sättigungs- und Kompetitionsassays mit mAChR beschrieben. Darüberhinaus wurde die Affinität von symmetrischen Bispyridiniumverbindungen an der orthosterischen Bindungsstelle humaner muskarinischer Acetylcholinrezeptoren des Subtyps M_5 untersucht.

Als Reporterligand wurde der Subtyp-unspezifische Antagonist [^{3}H]N-Methylscopolamin, der die orthosterische Bindungsstelle adressiert, eingesetzt. Bei der Methodenentwicklung stellte sich heraus, dass höhere Inkubationstemperaturen zu einer schlechteren Affinität des Liganden [^{3}H]N-Methylscopolamin führten. Der orthosterische Ligand besetzt vorerst ein Vestibül, das sich nahe an der Oberfläche des Rezeptors befindet und rutscht dann in die orthosterische Bindungsstelle, die tiefer in der extrazellulären Domäne lokalisiert ist (Haga et al., 2012). Die Affinitätsabnahme bei

höheren Temperaturen könnte vermutlich mit einer Zunahme der Molekülbeweglichkeit erklärbar sein, so dass der Ligand bei höheren Temperaturen womöglich wieder aus dem Vestibül „hüpft“, anstatt in die orthosterische Bindungstasche zu schlüpfen. Da die Struktur aller fünf mAChR-Subtypen hoch konserviert ist (Kruse et al., 2012; Thal et al., 2016), könnte dieses Phänomen auch für den M_5-Subtyp zutreffen.

Bis auf MB442, das einen *n*-Pentyl-Linker enthält und dessen Pyridiniumringe unsubstituiert sind, binden alle hier untersuchten Bispyridiniumverbindungen mit Affinitäten im mikromolaren Bereich und sind somit nicht so hochaffin wie die Antagonisten Atropin oder Scopolamin. Aber auch für die Agonisten Oxotremorin M und Carbamoylcholin wurden Affinitäten im mikromolaren Größenbereich ermittelt.

Welche intrinsischen Aktivitäten mit den Affinitäten verbunden sind, müssen noch Funktionalitätsuntersuchungen mit mAChR klären, die beispielsweise die G-Protein-Aktivierung untersuchen (Ziegler et al., 2011; Schrage et al., 2014). Die für MB327 ermittelten Effekte in verschiedenen Organmodellen lassen auf eine antagonistische Wirkung schließen (Königer et al., 2013; Neumaier et al., 2016).

3.5.3. Struktur-Wirkungsbeziehungen bei der Affinität an nAChR

Niessen, K.V., Seeger, T., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Thiermann, H., Worek, F. (2013): Affinities of bispyridinium non-oxime compounds to [^{3}H]epibatidine binding sites of *Torpedo californica* nicotinic acetylcholine receptors depend on linker length
Chem. Biol. Interact. **206**, 545-554

In der dritten Publikation wird beschrieben, wie sich die Länge der zwischen den beiden Pyridiniumringen befindlichen Alkylkette und das Substitutionsmuster symmetrischer Bispyridiniumverbindungen auf die Affinität zum Muskeltyp-nAChR (*Torpdo californica*) auswirkt. Zusätzlich wurde die Auswirkung auf die Aktivität der humanen erythrozytären AChE verglichen.

Unsubstituierte Bispyridiniumverbindungen mit einer Linkerlänge $\geq$ 5 C-Atomen inhibieren die [^{3}H]Epibatidinbindung in einer klassischen Kompetitionskurve und mit Affinitäten im mittleren mikromolaren Konzentrationsbereich ($pK_i \approx 5$), die mit zunehmender Länge des Alkyl-Linkers steigt. Die Anwesenheit divalenter Kationen (Ca^{2+}, Mg^{2+}) führte zu einer leichten Erhöhung der Affinität. Dies könnte an membranstabilisierenden Effekten liegen oder auch rezeptorvermitteltet sein. Beispielsweise wurde für den α7-nAChR eine direkte, modulatorische Interaktion mit divalenten Kationen beschrieben, die für orthosterische Liganden zu einer Affinitätssteigerung führt (Galzi et al., 1996; McLaughlin et al., 2009). Die Inhibition der humanen AChE-Aktivität zeigte ebenfalls eine Abhängigkeit von der chemischen Struktur: Je länger die Alkylkette der Bispyridiniumverbindung war, desto mehr wurde die Enzymaktivität gehemmt.

Auch 4-*tert*-Butyl substituierte Bispyridiniumverbindungen interagierten mit steigender Linker-Länge mit höherer Affinität an den [^{3}H]Epibatidin-Bindungsstellen. Im Vergleich zu den unsubstituierten Strukturanaloga war die Affinität etwas schwächer. Der Inhibition der AChE-Aktivität war dagegen stärker ausgeprägt.

War die Linkerlänge kürzer (C3 bis C4 je nach Substitution), war sowohl bei den unsubstituierten als auch bei den 4-*tert*-Butyl-substituierten Bispyridiniumverbindungen keine klassische Kompetition des Reporterligands zu beobachten. Vielmehr waren Affinitätszunahmen des Reporterliganden [^{3}H]Epibatidin feststellbar, die auf eine allosterische Interaktion am nAChR schließen lassen. Dieses Verhalten war bei den 4-*tert*-Butyl-substituierten Bispyridiniumverbindungen stärker ausgeprägt als bei den unsubstituierten Strukturanaloga. Darüberhinaus war eine nennenswerte Hemmung der AChE-Aktivität nicht feststellbar.

Insbesondere die Gesamtgröße der Bispyridiniumverbindungen spielt eindeutig eine Rolle, in welcher Art und Weise die Interaktion an den [^{3}H]Epibatidinbindungsstellen geschieht. Hinsichtlich der Affinität am Muskeltyp-nAChR konnten erste Struktur-Wirkungsbeziehungen aufgedeckt werden. Auch waren Korrelationen zur Hemmaktivität an der humanen AChE ersichtlich.

3.5.4. Funktionale Effekte an nAChR

Niessen, K.V., Muschik, S., Langguth, F., Rappenglück, S., Seeger, T., Thiermann, H., Worek, F. (2016): Functional analysis of *Torpedo californica* nicotinic acetylcholine receptors in multiple activation states by SSM-based electrophysiology
Toxicol. Lett. **247**, 1-10

Die vierte Publikation beschreibt die Auswirkung ausgewählter Bispyridiniumverbindungen auf die Funktionalität von nikotinischen Acetylcholinrezeptoren des Muskeltyps (*Torpedo californica*). Grundlegend in der Methode ist die gezielte Induzierung der Rezeptorzustände „aktiv“, „ruhend“, „desensitisiert“ und „resensitisiert“ (Abb. 7). Die hierfür entwickelte Methode mit verschiedenen Setups und auf Grundlage der SSM-basierten Elektrophysiologie (Schulz et al., 2008; Schulz et al., 2009), einer neuartigen Bilayer-Technik, wurde weltweit erstmals publiziert.

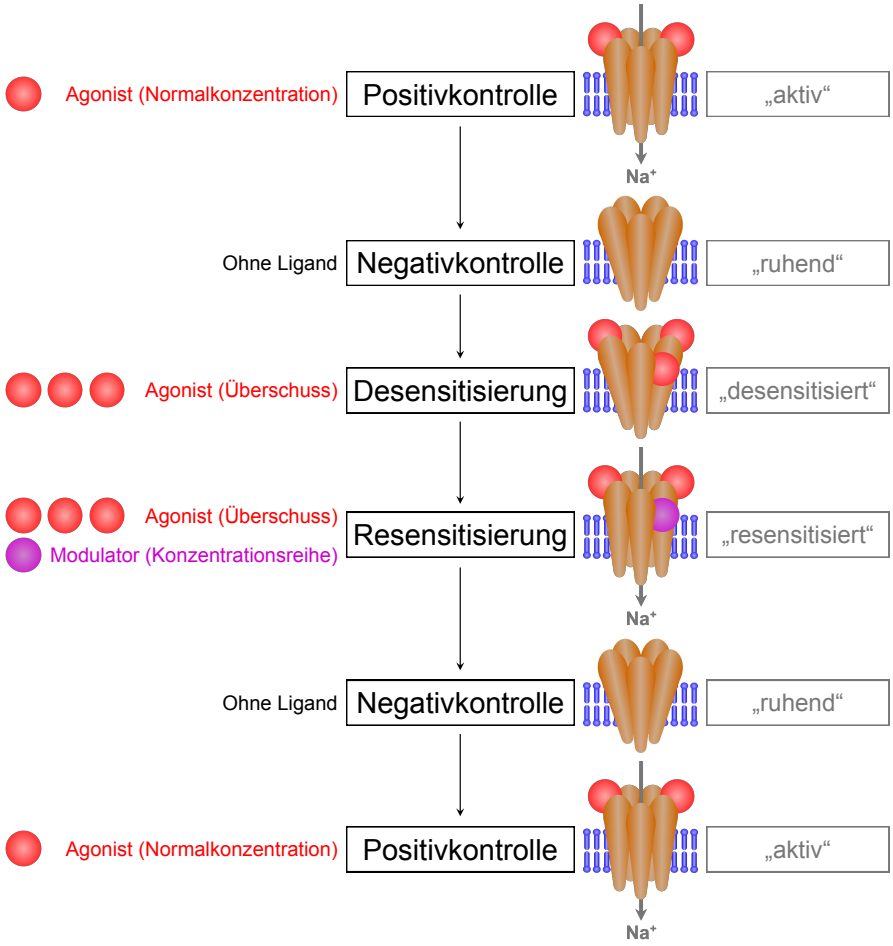


Abb. 7: Schematische Aufzeichnung einer Abfolge im Setup „Recovery“, die in einem einzelnen Sensor ablief. Je nachdem, ob und welche Liganden in welcher Konzentration vorhanden waren, wurden verschiedene Rezeptorzustände stabilisiert. Bei diesem mehrmaligen Durchlauf erhöhte sich die Konzentration des Modulators (= zu testende Substanz) um jeweils eine Zehnerpotenz. Als Positivkontrolle wurde das cholinerge Signal definiert, bei dem noch keine Desensitisierung zu beobachten war, als Negativkontrolle das Signal in Abwesenheit jeglicher Liganden. Für eine komplette Desensitisierung wurde der Agonist in ausreichendem Überschuss eingesetzt.

Als Agonist wurde Carbamoylcholin eingesetzt, das mit Acetylcholin strukturähnlich ist, aber im Gegensatz zu diesem nicht so stark hydrolyseempfindlich ist. Somit konnten auch längere Assays (z.B. über Nacht) durchgeführt werden, ohne dass befürchtet werden musste, dass sich die Konzentration des Agonisten aufgrund der Hydrolyse kontinuierlich verringerte.

Das Strukturanalogon MB779, das eine Alkylkette mit 10 C-Atomen aufweist (*n*-Decyl-Linker), zeigte eine antagonistische Wirkung: mit zunehmenden Konzentrationen wurde das cholinerge Signal abgeschwächt. Auch Pancuronium antagonisierte das Signal, jedoch in deutlich stärkerem Ausmaß.

In den Kompetitionsassays unter Einsatz des Agonisten [^{3}H]Epibatidin führten MB583, MB327 und MB781 zu einer Verstärkung der Affinität des radioaktiven Reporterliganden, die erst bei höheren Konzentrationen inhibiert wurde. Die langkettige Bispyridinium-verbindung MB779 zeigte dagegen eine klassische Kompetitionskurve (vergleiche dritte

Publikation). Die hierbei vermuteten positiv allosterischen (MB327, MB583, MB781) bzw. antagonistischen Wirkungen (MB779) von Bispyridiniumverbindungen konnten bei diesen vier ausgewählten Strukturen erstmalig mit dieser Methode, die direkt die funktionale Auswirkung einer Rezeptor-Ligand-Interaktion untersucht, bestätigt werden.

Es wurde nachgewiesen, dass MB327 nicht nur cholinerge Signale verstärken kann, sondern auch eine Agonist-induzierte Desensitisierung abhängig von seiner Konzentration wieder aufheben („resensitisieren“) kann. Die homologen Strukturen MB583 (Ethyl-Linker) und MB781 (*n*-Butyl-Linker) zeigten ebenfalls diese Eigenschaft, wenngleich schwächer als MB327 (*n*-Propyl-Linker). Dass nicht nur Signale von Agonisten verstärkt, sondern auch eine Desensitisierung wieder aufgehoben werden kann, ist eine typische Eigenschaft sogenannter „positiv allosterischer Modulatoren des Typs II“ (Typ II PAM) (Chatzidaki und Millar, 2015).

Substanzen, die als Typ II PAM am nAChR wirken, sind daher aussichtsreiche Kandidaten für die Entwicklung von Antidoten. Für das Screening großer Substanzbibliotheken wurden später die Funktionalitätsnachweise auf das Setup „Recovery“ (Abb. 7) beschränkt (vergleiche fünfte Publikation).

3.5.5. Vergleichende pharmakologische Charakterisierung

Niessen, K.V., Seeger, T., Rappenglück, S., Wein, T., Höfner, G., Wanner, K.T., Thiermann, H., Worek, F. (2018): *In vitro* pharmacological characterization of the bispyridinium non-oxime compound MB327 and its 2- and 3-regioisomers
Toxicol. Lett. **293**, 190-197

Die fünfte Publikation beschreibt, ob der nAChR-vermittelte Effekt des MB327 auch für die beiden Regioisomere PTM0001 und PTM0002 zutreffend ist. Bei deren pharmakologischen Charakterisierung werden hierbei die Affinität und funktionale Auswirkung am nAChR sowie der Effekt auf Soman-vergiftetes Muskelgewebe aufgezeigt.

Keine der drei Regioisomere zeigte eine „klassische“ Kompetitionskurve. Wie schon früher beschrieben (siehe Abschnitte 3.5.1 und 3.5.3) sind die hierbei beobachteten Affinitätszunahmen des orthosterischen Reporterliganden [^{3}H]Epibatidin allosterischen Effekten zuzuschreiben.

Die Wiederherstellung der Funktionalität desensitisierter nAChR fiel bei MB327 und PTM0001 etwa gleich aus, bei PTM0002 jedoch signifikant niedriger. Die hohe Anzahl an Bindungsstellen erforderte eine sehr hohe Konzentration an Carbamoylcholin, um eine vollständige Desensitisierung des nAChR herbei zu führen. Bedingt durch die limitierte Löslichkeit konnten für MB327, PTM0001 und PTM0002 maximal 1-10 mM eingesetzt werden, so dass nicht das komplette Konzentrationssprektrum (z.B. 10fach, 100fach oder 1000fach höhrere Konzentration im Vergleich zu Carbamoylcholin) betrachtet werden konnte.

Bei den Muskelkraftmessungen am Ratten-Diaphragma-Modell fiel auf, dass alle drei Regioisomere das gleiche Muster zeigten. Höhere Konzentrationen (> 300 µM) der hier untersuchten Bispyridiniumverbindungen führten dazu, dass sich das Ausmaß der Muskelkraftherstellung verringerte. Bei höheren Stimulationsfrequenzen, die mit stärkeren Ausschüttungen von Acetylcholin in den synaptischen Spalt verbunden sind, kam es zu keiner Rechtsverschiebung, d.h. Verschiebung des reaktivierenden Effekts zu höheren Konzentrationen.

Dieses Bild könnte eventuell mit der Hypothese, dass für die Bispyridiniumverbindungen mindestens zwei unterschiedlich lokalisierte Bindungsstellen existieren, erklärt werden. Eine Bindungsstelle hat eine höhere Affinität für die Bispyridiniumverbindungen, wird aber auch noch von dem endogenen Liganden Acetylcholin addressiert. Die andere Bindungsstelle weist eine niedrigere Affinität für die Bispyridiniumverbindungen auf, wird aber nicht von Acetylcholin besetzt (Abb. 8).

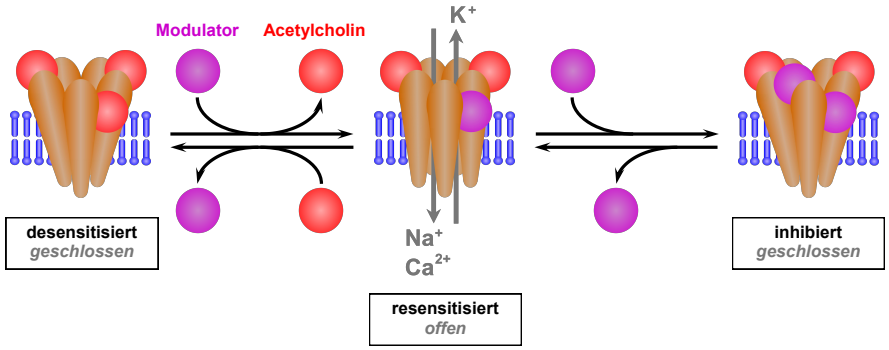


Abb. 8: Hypothese für die Erklärung positiv allosterischer und inhibitorischer Effekte der Bispyridiniumverbindungen MB327, PTM0001 und PTM0002 (als „Modulator“ bezeichnet). Eine höher affine, modulatorische Bindungsstelle wird von den Modulatoren (lila) und vom endogenen Acetylcholin (rot) besetzt und eine weitere niedrig affine, inhibitorische nur vom Modulator. Die Bispyridiniumverbindungen interagieren demnach als PAM und bei höheren Konzentrationen zusätzlich als NAM. Das endogene Acetylcholin interagiert als Agonist und bei höheren Konzentrationen zusätzlich als SAM („Desensitisierer“).

Auf Grundlage des Massenwirkungsgesetzes würde dies die beiden überlagerten Effekte erklären:

- Werden die Bispyridiniumverbindungen bis zu 300 µM appliziert, sinkt bei Erhöhung der Acetylcholinkonzentration (Erhöhung der Stimulationsfrequenzen) das Ausmaß der Muskelkraftwiederherstellung.

- Werden die Bispyridiniumverbindungen in höheren Konzentrationen (> 300 µM) appliziert, kommt es unabhängig von der Konzentration des Neurotransmitters zu einer Hemmung, die sich in einer verringerten Muskelkraftwiederherstellung äußert.

Die Überlagerung positiv modulatorischer und inhibitorischer Effekte in Abhängigkeit der Konzentration wurde auch bei einigen Bispyridiniumverbindungen mittels Patch Clamp-

Versuchen mit CHO-Zellen, die den humanen α7-nAChR stabil exprimieren, festgestellt (Scheffel et al., 2018a; Scheffel et al., 2018b). Kürzlich wurden mit Hilfe von *in silico*-Modellen des Muskeltyp-nAChR zwei mögliche MB327-Bindungsstellen identifiziert (Wein et al., 2018), die gegebenenfalls mit der oben beschriebenen Hypothese in Zusammenhang gebracht werden könnten.

Eine Ermüdung der Muskeln über die Zeitdauer des Experimentes konnte durch viele Vorversuche ausgeschlossen werden. Außerdem hätten etwaige Ermüdungserscheinungen inkonsistentere Bilder ergeben.

Wenngleich die Effekte an Modellen unterschiedlicher Komplexität (Proteinsuspension, Gewebe) und verschiedene Ebenen der neuromuskulären Transmission betrachet wurden, ergaben sie ein gemeinsames Bild, das die Hypothese der beiden oben beschriebenen Bindungsstellen stützen würde. Zum Beweis müssten aber die Bindungsstellen endgültig identifiziert und mit Affinitäts- und Funktionalitätsexperimenten charakterisiert werden.

3.5.6. Ergebnisse

Mit den hier eingesetzten Methoden konnten die vorhin genannten Fragestellungen weitgehend beantwortet werden:

- Abhängig von ihrer Struktur interagieren Bispyridiniumverbindungen direkt mit *Torpedo*-Muskeltyp- und humanen α7 nAChR.
- Bei der Interaktion mit Muskeltyp-nAChR (*Torpedo californica*) zeigen Bispyridiniumverbindungen mit einer kurzen Linkerlänge (≤ 5C) allosterische Effekte, die sich als eine Affinitätszunahme des orthosterischen Agonisten [^{3}H]Epibatidin äußern. Strukturanaloga mit längeren Alkyl-Linkern inhibieren die Bindung des orthosterischen Liganden [^{3}H]Epibatidin, was als klassische Kompetitionskurve in Erscheinung tritt. Bispyridiniumverbindungen mit einer 4-*tert*-Butylgruppe zeigen die allosterischen Effekte in stärkerem Ausmaß. Zudem hemmen Bispyridiniumverbindungen mit einem langen Alkyl-Linker die Aktivität humaner erythrozytärer AChE.
- Die Bispyridiniumverbindungen binden auch an mAChR. An humanen mAChR (Subtyp M_5) wird eine Affinität nachgewiesen, die in der Größenordnung von Agonisten liegt.
- 4-*tert*-Butylsubstituierte Bispyridiniumverbindungen mit kurzen Alkyl-Linkern (C2-C4) wirken als positiv allosterische Modulatoren, die in der Lage sind, den desensitisierten nAChR zu resensitisieren (Wirkung als Typ II PAM). Sind die Pyridiniumringe mit einem langen Alkyl-Linker (z.B. C10) verbunden, wirken die Bispyridiniumverbindungen inhibitorisch.

- Nach einer Soman-Vergiftung lässt sich die Muskelkraft von Ratten-Diaphragma-Hemisphären nicht nur mit MB327, sondern auch mit dessen 2- und 3-Regioisomeren PTM0002 und PTM0001 wiederherstellen. Die in verschiedenen Modellen gewonnenen Ergebnisse zeigen, dass die Wirkung tatsächlich rezeptorvermittelt ist und dem Wirkprofil eines Typ II PAM („Resensitizer“) entspricht. Jedoch sind bei höheren Konzentrationen ($>$ 300 µM) inhibitorische Effekte erkennbar, die hypothetisch mit der Interaktion an zwei verschiedenen Bindungsstellen erklärbar sein könnten.

Von den hier untersuchten, positiv pharmakologisch wirksamen Verbindungen ist keine wesentlich potenter und – soweit getestet – selektiver als MB327, dessen Wirkung bereits in Tierversuchen evaluiert wurde. Es müssen noch wirksamere Verbindungen identifiziert werden. Nichtsdestoweniger konnten wichtige Beiträge für die Entwicklung selektiverer und potenterer Wirkstoffe, die für die Behandlung von Nervenkampfstoff- oder Pestizidvergiftungen geeignet sind, erzielt werden.

4. Publikationen

4.1. Publikation I: *Toxicol. Lett.* 206 (2011), 100-104

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Interaction of bispyridinium compounds with the orthosteric binding site of human α7 and *Torpedo californica* nicotinic acetylcholine receptors (nAChRs)

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ABSTRACT

Standard treatment of poisoning by organophosphorus (OP) nerve agents with atropine and oximes lacks efficacy with different nerve agents. A direct pharmacologic intervention at the nicotinic acetylcholine receptor (nAChR) was proposed as an alternative therapeutic approach and promising in vitro and in vivo results were obtained with the bispyridinium compound SAD-128. In addition, a number of SAD-128 analogues improved neuromuscular transmission of soman-poisoned diaphragms in vitro. We investigated the interaction of six of these SAD-128 analogues with the orthosteric binding site of the human α7 nAChR and *Torpedo californica* nAChR with a high-throughput assay using radioactive ligands. The determined affinity constants indicate a weak interaction of three test compounds (K_i in the micromolar range) with both receptors, but no interaction could be recorded with the other three test compounds. The six SAD-128 analogues showed a low intrinsic inhibitory potency with human acetylcholinesterase (IC_{50} > 400 μM). In conclusion, the results of the present study do not indicate a correlation between the affinity to the orthosteric binding site and the functional improvement of neuromuscular transmission and it is assumed that other mechanisms contribute to the therapeutic effect of the tested compounds.

1. Introduction

Organophosphorus (OP) nerve agents inhibit the pivotal enzyme acetylcholinesterase (AChE) irreversibly. Inhibition of AChE in the synaptic cleft leads to accumulation of acetylcholine (ACh) and subsequent overstimulation of central and peripheral muscarinic (mAChR) and nicotinic (nAChR) receptors. The resultant cholinergic syndrome is characterized by disturbance of numerous body functions and may lead finally to central and peripheral respiratory failure and death.

Standard treatment of nerve agent poisoning includes the administration of atropine as an anticholinergic agent and oximes, e.g., obidoxime or pralidoxime, as AChE reactivators (Eyer and Worek, 2007).

At therapeutic concentrations atropine acts exclusively as a reversible antagonist at mAChRs while oximes may provide a causal treatment by reactivating OP-inhibited AChE which is of utmost importance to restore neuromuscular transmission at respiratory muscles (Thiermann et al., 2010). Clinically used and experimental oximes were shown to be potent reactivators of AChE inhibited by different nerve agents and pesticides but lack efficacy with tabun- and soman-inhibited AChE (Worek et al., 2004).

In order to overcome the limited therapeutic efficacy of oximes in cases of poisoning by different nerve agents a direct, pharmacologic intervention at nAChRs was proposed as a new therapeutic approach to improve nerve agent-impaired neuromuscular transmission (Sheridan et al., 2005).

Previous studies with the bispyridinium non-oxime SAD-128 demonstrated its therapeutic effect against soman in vitro and in vivo which was partly attributed to its interaction with nAChRs (Schoene and Oldiges, 1973; Harris et al., 1977; Štalc and Šentjurc, 1990; Alkondon and Albuquerque, 1989; Grubič and Tomažič, 1989).

In vertebrates, nAChRs mediate synaptic transmission at the skeletal neuromuscular junction. The muscle-type ($\alpha1\beta1\delta\varepsilon$) nAChRs show a high degree of homology with *Torpedo* nAChRs ($\alpha\beta\delta\gamma$) (Millar, 2003) and the α7 nAChR subtype, which was originally classified as a neuronal receptor, appears to be widespread in the human body (Fagerlund and Eriksson, 2009).

In the present study, six novel SAD-128 analogues (Fig. 1) (Timperley et al., 2005), which showed promising effects in improving soman-impaired neuromuscular transmission (Turner, 2007),

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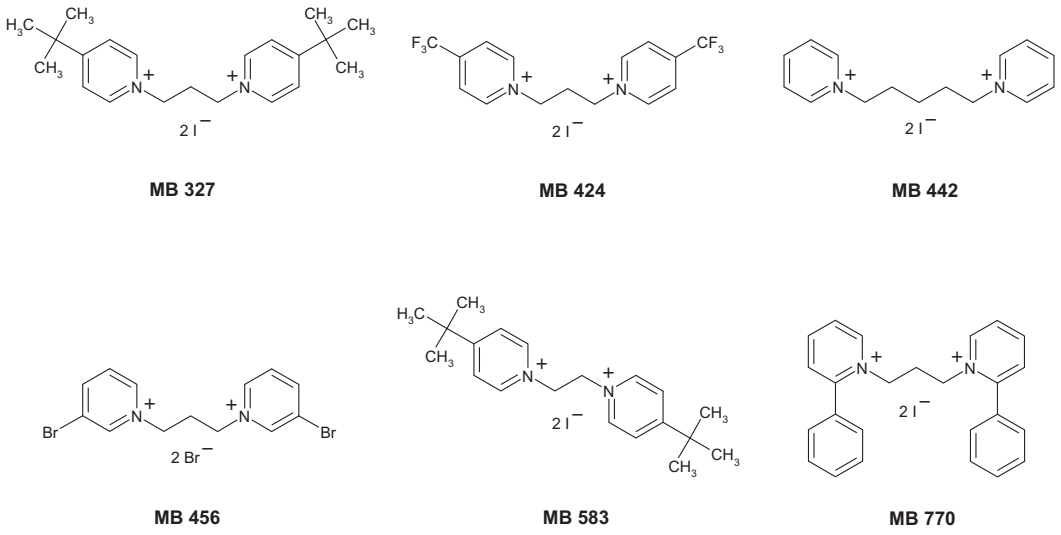

Fig. 1. Bispyridinium compounds with different alkyl chains and pyridinium-substituents.

were investigated to determine their interaction with the orthosteric binding site of nAChRs. The experiments were performed with two nAChR subtypes, the human homomeric α7 nAChR and the heteromeric αβδγ-nAChR isolated from *Torpedo californica* electric tissue applying a newly developed high-throughput binding assay.

2. Materials and methods

2.1. Materials

The GH_4C_1/hα7 nAChR cell line was obtained from Genionics, Schlieren, Switzerland. *T. californica* electroplaque tissue was purchased from Aquatic Research Consultants, San Pedro, CA, USA. Disposables and cell culture flasks were from Becton & Dickinson, Heidelberg, Germany, and Nunc, Thermo Scientific, Langenselbold, Germany. 500 cm^2 cell-culture plates were supplied by Corning, Amsterdam, The Netherlands, and the complete cell culture media and supplements were purchased from Gibco, distributed by Invitrogen, Darmstadt, Germany. Epibatidine, [5,6-cycloheptyl-^{3}H] with a specific activity of approx. 2 TBq/mol was from Perkin Elmer, Jügesheim, Germany. PNU 282987, α-bungarotoxin, methyllycaconitine and (±)-epibatidine were obtained from Tocris, Bristol, UK, and pancuronium, carbamoylcholine, acetylthiocholine iodide (ATCh) and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent) from Sigma–Aldrich, Taufkirchen, Germany.

The bispyridinium compounds MB442, MB583, MB327, MB770, MB456 and MB424 (Fig. 1) were synthesized according to Turner (2007) at Dstl Porton Down, UK.

Stock solutions of MB compounds, methyllycaconitine, pancuronium and carbamoylcholine were prepared in distilled water (10 mM), (±)-epibatidine and PNU 282987 in ethanol (10 mM), and α-bungarotoxin in 10% aqueous DMSO (1 mM).

2.2. Cell culture

GH_4C_1 cells, derived from a rat pituitary tumor cell line, were stably transfected with the human α7 nAChR (Genionics, Schlieren, Switzerland) and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/l glucose and GlutaMAX supplemented with 10% heat-inactivated fetal bovine serum and 100 μl/ml of the selective antibiotic, geneticin (G418) (Steiner et al., 2010). Cells were maintained at 37 °C and 5% CO_2/humidified air up to 70–80% confluence and split twice a week, using enzyme-free dissociation buffer for detachment.

2.3. Cell membrane preparation

GH_4C_1 cells, stably expressing the human α7-nAChR (GH_4C_1/hα7-nAChR cells), were grown to 70–80% confluence (~400 × 10^6 cells) in 500 cm^2 cell-culture plates. After removal of the cell-culture media, the cell monolayer was washed with ice-cold phosphate-buffered saline (4 mM phosphate and 150 mM NaCl), supplemented with 1 mM EDTA (PBS-EDTA) and 25 ml of PBS-EDTA buffer was added. Then, the cell-culture plates were incubated for 60 min at 4 °C to detach the cells which were then transferred into polypropylene tubes and subsequently centrifuged at 700 × *g* for 10 min and 4 °C to gain a pellet. The pellet was shock frozen in liquid nitrogen and stored at −80 °C until use.

All subsequent steps were conducted at 4 °C to avoid receptor degradation. The frozen pellets were thawed in a 10-fold volume of lysis buffer (1 mM NaH_2PO_4/Na_2HPO_4, 0.5 mM EDTA, pH 7.4), suspended by Dounce homogenisation (50 up and down strokes), and centrifuged at 1000 × *g* for 10 min. The supernatant was combined, mixed with a 5-fold volume of pellet buffer (10 mM NaH_2PO_4/Na_2HPO_4, 120 mM NaCl, 5 mM KCl, pH 7.4), and centrifuged at 100,000 × *g* for 30 min (Beckman ultracentrifuge). The supernatant was discarded and the pellet homogenised in storage buffer (10 mM NaH_2PO_4/Na_2HPO_4, 120 mM NaCl, 5 mM KCl, 300 mM sucrose, 0.5 mM EDTA, pH 7.4), corresponding to about 5 mg protein/ml. Total protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin as standard. Aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Preparation of (αβδγ) nAChR enriched membrane fragments

Membranes were prepared from frozen electric organ of *T. californica* (Aquatic Consultants, San Pedro, USA) as described before (Elliot et al., 1980) with minor modifications. All subsequent steps were consequently performed at 4 °C.

A threefold volume of extraction buffer (20 mM Na_2HPO_4/NaH_2PO_4, 400 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (freshly added), pH 7.4) was added to frozen tissue. After thawing, the mixture was dispersed with an Ultra-Turrax at 13,500 rpm. The homogenate was centrifuged for 90 min at 27,000 × *g* (Beckman ultracentrifuge). The pellet was resuspended in wash buffer (extraction buffer without NaCl) and centrifuged for 60 min at 37,000 × *g*. After three washes, the suspension was centrifuged for 10 min and 1000 × *g*. The supernatant was mixed with ≈5-fold volume of pellet buffer (10 mM Na_2HPO_4/NaH_2PO_4, 120 mM NaCl, 5 mM KCl, pH 7.4) and centrifuged for 30 min at 100,000 × *g*. The residue was suspended with a double volume of storage buffer (10 mM Na_2HPO_4/NaH_2PO_4, 120 mM NaCl, 5 mM KCl, 300 mM sucrose, 0.5 mM EDTA, pH 7.4), aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

2.5. Radioligand binding

Radioligand binding experiments were performed according to described [^{3}H] epibatidine assay methods (Quik et al., 1996) with few modifications.

Pipetting and incubation were carried out with a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany). All radioligand experiments were conducted in 96-deep well plates, in assay binding buffer (10 mM NaH_2PO_4/Na_2HPO_4, 120 mM NaCl, 5 mM KCl, pH 7,4). An aliquot of the GH_4C_1/hα7 nAChR membrane fraction was rapidly thawed and diluted in a fivefold volume of cold binding buffer. *Torpedo* membranes were 200-fold diluted with binding buffer, containing additional 0.2% bovine serum albumin. In the case of *Torpedo* nAChRs, total protein amount was 10 μg per well and with hα7 nAChRs 50 μg per well. Total volume in each well was 250 μl. The membrane suspension was stirred (200 rpm) at +4 °C. After the incubation period of 120 min at 25 °C, bound and free [^{3}H] epibatidine were separated by rapid vacuum filtration using a cell harvester (Perkin Elmer, Jügesheim, Germany) onto GF/B filter plates, previously pre-soaked in 0.1%

polyethylenimine (Bruns et al., 1983) and rapidly washed eight times with ice cold assay binding buffer. After 60 min drying at 50 °C, the membrane-containing filters were treated with a melt-on scintillator (MeltiLex B, Perkin Elmer-Wallac, Turku, Finland) for 4 min at 95 °C. Radioactivity was quantified using single photon counting on a MicroBeta scintillation counter (Perkin Elmer, Jügesheim, Germany) at ambient temperature. In all experiments, total ligand binding never exceeded more than 10% of the added ligands in order to limit complications associated with depletion of the free radioligand concentration (Rovati, 1993).

2.6. [^{3}H] Epibatidine saturation binding

Binding was performed with concentrations ranging from 10 pM to 10 nM [^{3}H] epibatidine to obtain saturation binding curves. Non specific binding of *Torpedo* nAChRs and hα7 nAChRs was determined in presence of 10 μM (±)-epibatidine and 10 μM PNU 282987, respectively. Aliquots of protein suspension (≈50 μg/well GH_4C_1/hα7 cell membranes, ≈1.5 μg/well *Torpedo* protein) were incubated at room temperature with gentle agitation (≈100 rpm) for 120 min.

2.7. Competition assays

To obtain the binding affinity of unlabelled compounds to the orthosteric binding site, [^{3}H] epibatidine competition experiments were performed at equilibrium. [^{3}H] Epibatidine was used at an assay concentration of 3 nM with *T. californica* membranes and 600 pM with GH_4C_1/hα7 membranes. Total binding was determined without competitor and non-specific binding in the presence of 10 μM unlabelled (±)-epibatidine in the assay. [^{3}H] Epibatidine was incubated in the presence of the indicated concentration of the unlabelled compounds and protein suspension (≈50 μg/well GH_4C_1/hα7 cell membranes, ≈1.5 μg/well *Torpedo* protein) at room temperature with gentle agitation for 120 min.

2.8. Data analysis

Specific binding was defined as the difference between total binding and non-specific binding. The concentration of a competing test compound that inhibited 50% of specific binding (IC_{50}) was calculated with nonlinear regression (Prism 5.0, GraphPad Software, San Diego, CA, USA) for sigmoidal dose–response curves obtained in competitive binding experiments (One site – Fit K_i). Top and bottom of the sigmoidal curve were constrained to values obtained for total binding (without competitor, top) or non-specific binding (in the presence of 10 μM (±)-epibatidine, bottom). K_i values were determined according to the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Values for the equilibrium dissociation constant (K_D) and the maximum density of binding sites (B_{max}) were calculated from binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 5.0).

All data are shown as means ± SEM (n = 3–6).

2.9. AChE activity

Hemoglobin-free human erythrocyte ghosts were used as the AChE source and were prepared according to Dodge et al. (1963) with minor modifications (Worek et al., 2002). Stock solutions (1.15 mM) of the bispyridinium compounds (Fig. 1) were prepared in phosphate buffer (0.1 M, pH 7.4) and were stored at −80 °C until use.

2.10. Inhibition of AChE by bispyridinium compounds

A slightly modified colorimetric Ellman assay (Worek et al., 1999; Eyer et al., 2003) was used for the determination of AChE activities (Cary 3Bio, Varian, Darmstadt, Germany) at 436 nm. The standard assay mixture (3.16 ml) contained 1.0 mM ATCh as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4). Assays were run at 37 °C.

The inhibitory potency of bispyridinium compounds, quantified as IC_{50} was determined by incubation of AChE with 10^{-6}–10^{-3} M test compound for 5 min before adding substrate to determine enzyme activity. AChE activities were referred to native controls (% of control). The IC_{50} was calculated from semi-logarithmic plots of the test compound concentration versus the AChE activity (sigmoidal-dose response, Prism 5.0).

3. Results

The two receptor membrane preparations had a very different maximum number of binding sites (B_{max}) that were occupied by [^{3}H] epibatidine. With GH_4C_1/hα7-nAChR membrane preparations B_{max} values of 204.1 ± 13.6 fmol/mg protein were reached, while with *Torpedo* nAChR a B_{max} 42.4 ± 9.6 pmol/mg protein was recorded. It should be noted that only the total number of binding sites without distinction between high and low affinities was determined. An [^{3}H] epibatidine equilibrium dissociation constant (K_D) of 4.6 ± 1.1 nM and 13.9 ± 4.8 nM was calculated for α7 nAChRs and *T. californica* nAChRs, respectively. Fig. 2 exemplifies saturation isotherms with GH_4C_1/hα7-nAChR membrane preparations.

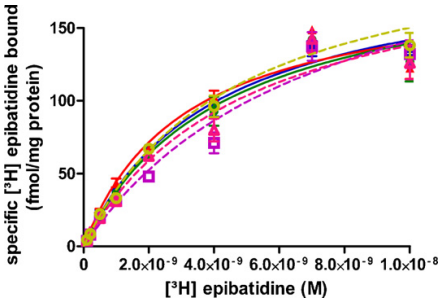

Fig. 2. Saturation isotherms of [^{3}H] epibatidine binding to GH_4C_1/hα7-nAChR membrane fractions. Results of 6 experiments each performed in triplicate and expressed as mean ± SEM.

Table 1
Interaction of ligands with decreasing affinities at [^{3}H] epibatidine binding sites of hα7-nAChR. K_i is the equilibrium dissociation constant, pK_i is the negative logarithm of K_i. Data are means ± SEM (n = 3). n.d. not detectable.

Ligand	K_i (nM)	pK_i
(±)-Epibatidine	2.0 ± 0.3	8.7 ± 0.1
PNU 282987	25.4 ± 0.5	7.6 ± 0.1
Methyllycaconitine	82.1 ± 9.8	7.1 ± 0.1
α-Bungarotoxin	1922 ± 1014	5.7 ± 0.2
Carbamoylcholine	9495 ± 2027	5.0 ± 0.1
MB770	15,101 ± 3850	4.8 ± 0.1
MB456	40,087 ± 37,364	4.4 ± 0.2
MB442	191,425 ± 161,820	3.7 ± 0.8
MB583	n.d.	n.d.
MB327	n.d.	n.d.
MB424	n.d.	n.d.

The binding affinities of the tested ligands for hα7 nAChRs showed huge differences (Table 1 and Fig. 3). The bispyridinium compounds MB770, MB456 and MB442 exhibited K_i values of 15 μM, 40 μM and 191 μM, respectively. With MB583, MB327 and MB424 no interaction with the orthosteric binding site was detected. With *Torpedo* nAChRs the affinity was higher with MB770 and MB442 (Table 2 and Fig. 4) and again no interaction of MB583, MB327 and MB424 with the orthosteric binding site could be detected.

The comparison of K_i values between hα7 and *Torpedo* nAChRs revealed in part substantial differences. A roughly 10-fold higher affinity of α-bungarotoxin and MB442 towards *Torpedo* nAChRs was recorded.

Carbamoylcholine, a muscarinic and nicotinic receptor agonist, bound to hα7 nAChRs in the same order of magnitude as

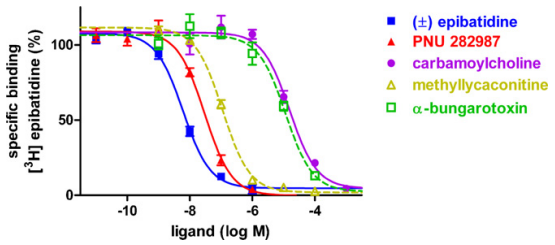

Fig. 3. Representative competition binding curves of series of similar assays with hα7-nAChR membrane fraction. Each data point results from six replicates (means ± SEM). Dashed line: antagonists, solid line: agonists.

Table 2
K_i and pK_i values in descending order at [^{3}H] epibatidine binding sites of *Torpedo*-nAChR. Data are means ± SEM (n = 3). n.d. not detectable, * data could not be analysed due to artefacts.

Ligand	K_i (nM)	pK_i
(±)-Epibatidine	4.8 ± 1.0	8.3 ± 0.1
Pancuronium	35.7 ± 10.3	7.4 ± 0.1
α-Bungarotoxin	199.6 ± 35.2	6.7 ± 0.1
MB770	7194 ± 1699	5.1 ± 0.1
MB442	15,704 ± 5625	4.8 ± 0.2
Methyllycaconitine	16,904 ± 12,888	4.8 ± 0.2
MB456	46,881 ± 24,379	4.3 ± 0.2
MB583	n.d.	n.d.
MB327	*	*
MB424	*	*

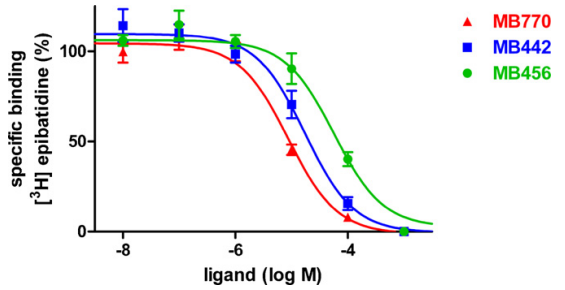

Fig. 4. Representative competition binding curves of a series of similar assays with *Torpedo* nAChR membrane fractions. Each data point represents six replicates (means ± SEM).

Table 3
Inhibition of human AChE by bispyridinium compounds. IC_{50} values in descending order. Data are means ± SEM (n = 2).

Compound	IC_{50} (mM)
MB456	0.42 ± 0.01
MB583	0.58 ± 0.03
MB327	0.60 ± 0.04
MB442	>1
MB770	>1
MB424	>1

α-bungarotoxin ($K_i \approx 9$ μM). Interestingly, an unexpected phenomenon was observed with carbamoylcholine, MB327 and MB424 and *Torpedo* nAChRs: with increasing concentrations of these compounds the specific binding of [^{3}H] epibatidine increased and reached a maximum of up to 400% of total binding at approx. 1 μM ligand and decreased at higher ligand concentrations. Due to this artefact no K_i values could be calculated.

The incubation of human AChE with the bispyridinium compounds resulted in IC_{50} values greater than 400 μM (MB456, Table 3). With MB442, MB770 and MB424 no IC_{50} could be determined at the given conditions. These data indicate a remarkably low intrinsic inhibitory potency of these compounds which is of importance for their use as therapeutic drugs.

4. Discussion

The present study presents data on the interaction between different nicotinic agonists and antagonists with hα7 nAChRs and *Torpedo* nAChRs applying an automated high-throughput assay and [^{3}H] epibatidine as radioactive marker. In comparison to manual procedures, the automated assay allows a 6 fold higher number of experiments at standardized and controlled conditions. Hereby, we detected a higher dissociation constant than previously determined with α-bungarotoxin and hα7 nAChRs or *Torpedo* nAChRs. α-Bungarotoxin is described as a potent antagonist that binds with a high affinity (K_i = 0.48 nM) to hα7 nAChRs (Gopalakrishnan et al., 1995) and *Torpedo* nAChRs (K_D = 0.23–0.60 nM) (Lukas et al., 1981). It seems to be crucial, whether an agonist such as [^{3}H] epibatidine or an antagonist like the conventional [^{125}I] α-bungarotoxin is used in competition assays with unlabelled α-bungarotoxin. This assumption is supported by experimental data with the agonist [^{3}H] nicotine, where a K_i value > 100 μM was found, while the competition between [^{3}H] methyllycaconitine and α-bungarotoxin resulted in K_i values ~7 nM (Ayers et al., 2002). Hence, this phenomenon could explain the relatively low K_i value of α-bungarotoxin with hα7 nAChRs (≈2 μM) and *Torpedo* nAChRs (~200 nM) if [^{3}H] epibatidine was used as radioactive tracer. Correspondingly, different K_i values were recorded for pancuronium, PNU 282987 and carbamoylcholine under these conditions compared to α-bungarotoxin (Gopalakrishnan et al., 1995; Gerzanich et al., 1995; Macallan et al., 1988). This confirms that epibatidine and α-bungarotoxin do not bind in the same way to orthosteric binding sites (Kawai et al., 2008).

The results of the present study demonstrate a moderate affinity of three bispyridinium compounds, MB770, MB442 and MB456, to the orthosteric binding site of hα7 and *Torpedo* nAChRs while three other compounds, MB583, MB327 and MB424, did not show any interaction. A recent in vitro study with soman-poisoned guinea pig diaphragm demonstrated a significant therapeutic effect of MB442, MB583, MB327, MB770 and MB456 at concentrations of 30–100 μM (Turner, 2007). Obviously, with these compounds no correlation between the affinity to the orthosteric binding site and the functional improvement of neuromuscular transmission exists, although species-related differences cannot be excluded.

Nevertheless, an interaction of these compounds with nicotinic receptors may contribute to the therapeutic effect. This may be assumed from the finding that MB327 enhances [^{3}H] epibatidine binding to the heteromeric *Torpedo* nAChR at a distinct concentration range which may possibly suggest an allosteric effect, where binding sites that were previously inaccessible are opened (Arias et al., 2009). Such an effect could not be observed with other test compounds.

A crucial aspect for the evaluation of the tested bispyridinium compounds as potential therapeutic drugs against OP poisoning is the intrinsic inhibitory potency towards AChE. Fortunately, the investigated compounds inhibited human AChE either at rather high concentrations (MB456, MB583, MB327) or were totally inactive (MB442, MB770, MB424).

In conclusion, an improved high-throughput receptor binding assay was applied to investigate the interaction of six novel SAD-128 analogues with the orthosteric binding site of hα7 nAChRs and *Torpedo* nAChRs. Under these conditions, three of the six investigated compounds interacted with the [^{3}H] epibatidine binding site at micromolar concentrations.

Conflicts of interest

There are no conflicts of interest.

Acknowledgements

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Competition radioligand binding assays for the investigation of bispyridinium compound affinities to the human muscarinic acetylcholine receptor subtype 5 (hM_5)

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Standard treatment of poisoning by organophosphorus (OP) nerve agents with atropine and oximes lacks efficacy with some nerve agents. Promising *in vitro* and *in vivo* results were obtained with the bispyridinium compound SAD-128 which was partly attributed to its interaction with nicotinic acetylcholine receptors. Previous studies indicate that bispyridinium compounds interact with muscarinic acetylcholine receptors as well. The muscarinic M_5 receptor is not well investigated compared to other subtypes, but could be important in the search for new drugs for treating nerve agent poisoning. A set of bispyridinium compounds structurally related to SAD-128 were tested in competition binding experiments with recombinant human M_5 muscarinic acetylcholine receptors. Five of the six investigated bispyridinium compounds interacted with the orthosteric binding site, with affinities in the low micromolar range. These data indicate that interaction of bispyridinium compounds with muscarinic receptors may contribute to their therapeutic efficacy. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: muscarinic acetylcholine receptor; human M_5 receptor; bispyridinium compound; radioligand binding assay; [^{3}H] N-methylscopolamine

Introduction

Muscarinic receptors responding to the natural ligand acetylcholine have a widespread tissue distribution and are involved in the control of numerous central and peripheral responses. They belong to the group of G-protein coupled receptors (GPCRs) because they are mediated by guanine nucleotide-dependent transducer proteins, the so-called G-proteins. M_2 and M_4 muscarinic receptor subtypes can couple to $G_{i/o}$ proteins, and M_1, M_3 and M_5 subtypes to $G_{q/11}$ proteins.[1] Coupling to the G-protein causes the receptors to convert to a high-affinity state for agonists.[2] The orthosteric binding pocket of the muscarinic receptor family is highly conserved, making the development of subtype selective agonists and antagonists very difficult.[3] The function of the M_5 mAChR is not yet clear, because the inability to clearly distinguish it from subtype M_3 has led to confusion about its physiological role. Determination of the precise distribution of M_5 receptors within different tissues is complicated by inadequate selectivity of radioligands as well as the low sensitivity/selectivity of polyclonal antisera in immunocytochemical studies. It appears that muscarinic M_5 receptors are selectively enriched in the *Substantia nigra* and ventral tegmental areas of the brain, suggesting that they may have a role in the modulation of dopaminergic transmission,[4] and stimulation of M_5 AChR results in an activation of dopaminergic pathways.[5] It has also been shown, however, that M_5 mAChRs are expressed ubiquitously throughout the brain and in non-neuronal tissues.[6] For example, the M_5 mAChR is required for cholinergic dilation of central blood arteries and arterioles.[7]

The role of M_5 mAChR in context of intoxication with organophosphorus (OP) nerve agents is not well investigated so far. Exposure to organophosphorus compounds has been associated with down-regulation of M_5 AChR related genes. Stimulation of M_5 AChR results in an activation of dopaminergic pathways which might explain the relationship between OP exposure and higher incidence of Parkinson's disease.[5] Although the physiological role of this receptor subtype has not been mapped comprehensively, investigations in the context of drug discovery for the treatment of nerve agent poisoning should not be neglected. Previous studies with the bispyridinium non-oxime SAD-128 demonstrated its therapeutic effect against soman *in vitro* and *in vivo* which was

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Abbreviations: *mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; GPCR, G-protein-coupled receptor; CHO, Chinese Hamster Ovary; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; AQ-RA 741, 11-[[4-[4-(Diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one; 4-DAMP, 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide; NMS, N-methylscopolamine methyl chloride; VU 0238429, 1-(4-Methoxybenzyl)-5-trifluoromethoxyisatin*

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partly attributed to its interaction with nicotinic acetylcholine receptors (nAChRs).[8–13] In addition, bispyridinium compounds have been shown to interact with muscarinic acetylcholine receptors, although primarily with the allosteric binding site.[14–18] In comparison to other mAChR subtypes, only a few binding studies have been performed with human M_5 mAChRs. Therefore, we initiated the present study to investigate interactions of non-oxime bispyridinium compounds with the M_5 mAChR subtype and focused on the orthosteric binding site. For the pharmacological characterization of this binding site, receptor binding experiments with hM_5 stably expressed in CHO cells were for example, phosphate buffer,[19–21] Tris–HCl buffer,[22] Krebs-Henseleit buffer,[23] and HEPES buffer,[24–27] and beyond the incubation time (1–5 h) and temperature (20-37 °C). In this present work, receptor binding experiments with [^{3}H]NMS as marker and 10 mM HEPES buffer as assay buffer were conducted. As an integral part of study, six novel SAD-128 analogues (Figure 1),[28] which showed promising effects in improving soman-impaired neuromuscular transmission,[29] were tested on membrane preparations of hM_5 mAChR/CHO-K1 cells applying a high-throughput binding assay with a custom-designed pipetting platform.

Materials and methods

Materials

CHO-K1 cell membrane preparations with human muscarinic receptors subtype M_5, and scopolamine methyl chloride, [N-methyl-^{3}H] with a specific activity of approximately 3 TBq/mmol, were purchased from Perkin Elmer, Jügesheim, Germany. AQ-RA 741, 4-DAMP, 5-methylfurmethiodide, oxotremorine M, pirenzepine, scopolamine hydrobromide and VU 0238429 were obtained from Tocris, Bristol, UK, atropine and carbamoylcholine chloride from Sigma-Aldrich, Taufkirchen, Germany. The bispyridinium compounds MB327, MB424, MB442, MB456, MB583 and MB770 (Figure 1) were synthesized at Dstl Porton Down, Salisbury, UK.[29] Stock solutions of bispyridinium compounds (1 mM), scopolamine hydrobromide (1 mM) and carbamoylcholine chloride (10 mM) were prepared in distilled water, atropine in ethanol (10 mM), and AQ-RA 741, 4-DAMP, 5-methylfurmethiodide, oxotremorine M and pirenzepine in 10% aqueous DMSO (1 mM), VU 0238429 in DMSO (1 mM) and stored at −20 °C until use.

Radioligand binding

Radioligand binding experiments were performed according to described [^{3}H]NMS assay methods[30] with a few modifications. Pipetting and incubation were carried out with a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany). All radioligand experiments were conducted in 96-well plates, in assay binding buffer (10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 adjusted with NaOH at ambient temperature). An aliquot of the CHO/hM_5 mAChR membrane fraction was rapidly thawed and diluted in a 50-fold volume of cold binding buffer. Total protein amounted to 10 µg per well and total volume in each well was 250 µl. The membrane suspension was stirred (200 rpm) at +4 °C. After an incubation period of 120 min at 20 °C, bound and free [^{3}H]NMS were separated by rapid vacuum filtration using a cell harvester (Perkin Elmer, Jügesheim, Germany) onto GF/B filter plates (Perkin Elmer, Jügesheim, Germany), previously pre-soaked for 10 min in 0.1% polyethylenimine[31] to minimize non-specific binding[32] and rapidly washed eight times with ice-cold washing buffer (10 mM HEPES, pH 7,4 adjusted with NaOH at 1 °C). After 60 min drying at 50 °C, the membrane-containing filters were treated with a melt-on scintillator (MeltiLex B, Perkin Elmer-Wallac, Turku, Finland) for 4 min at 95 °C. Radioactivity was quantified using single photon counting on a MicroBeta scintillation counter (Perkin Elmer, Jügesheim, Germany) at ambient temperature. For considering the quench effects, concentrations of bound [^{3}H]NMS were calculated with a calibration curve (linear regression). To obtain the calibration curve, aliquots of the [^{3}H]NMS concentrations used in the assay were applied to the filter mate before melting of the solid scintillator.

In all experiments, total ligand binding never exceeded more than 10% of the added ligands in order to limit complications associated with depletion of the free radioligand concentration.[33,34]

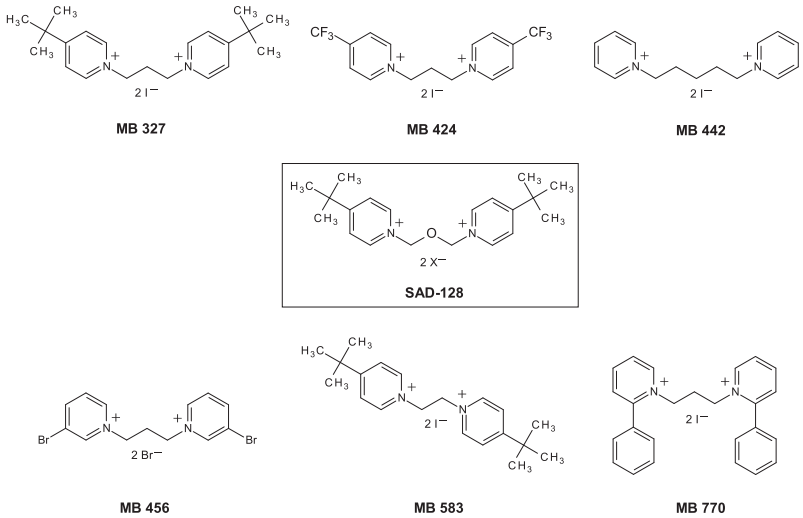

Figure 1. Structures of the tested bispyridinium compounds which are structurally related to SAD-128.

Total protein concentration was determined by the bicinchoninic acid method,[35] using bovine serum albumin as standard.

[³H]NMS saturation binding

Binding was performed with assay concentrations of 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 4 nM, 7 nM and 10 nM [³H]NMS to obtain saturation binding curves. Non-specific binding was determined in the presence of 10 µM atropine. Aliquots of protein suspension (≈ 10 µg/well) were incubated at 20 °C with gentle agitation (≈ 300 rpm) for 120 min. Binding data for each concentration were obtained from triplicates.

Competition assays

To obtain the binding affinity of unlabelled compounds to the orthosteric binding site, [³H]NMS competition experiments were performed at equilibrium. [³H]NMS was used at an assay concentration of 3 nM. Total binding was determined without competitor and non-specific binding in the presence of 10 µM unlabelled atropine in the assay. [³H]NMS was incubated in the presence of six indicated concentrations of the unlabelled compounds and protein suspension (≈ 10 µg/well) at 20 °C with gentle agitation for 120 min. The estimation of K_i values included three independent experiments, each performed in six replicates.

Data analysis

Specific binding was defined as the difference between total binding and non-specific binding. The concentration of a competing test compound that inhibited 50% of specific binding (IC_{50}) was calculated with nonlinear regression (Prism 5.0, GraphPad Software, San Diego, CA, USA) for sigmoidal dose–response curves obtained in competitive binding experiments (One site – Fit K_i). Top and bottom of the sigmoidal curve were constrained to values obtained for total binding (without competitor, top) or non-specific binding (in the presence of 10 µM atropine, bottom). K_i values were determined according to the Cheng and Prusoff equation.[36] Values for the equilibrium dissociation constant (K_D) and the maximum density of binding sites (B_{max}) were calculated from binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 5.0). All data are expressed as means ± SEM (n = 3–6).

Results and discussion

Saturation assays

Depending on the incubation temperature, the membrane preparation revealed a slightly different maximum number of binding sites (B_{max}) that were occupied by mAChR antagonist [³H]NMS which addressed the orthosteric binding site. Figure 2 shows saturation isotherms with assay temperatures adjusted to 20 °C (Figure 2a) and 30 °C (Figure 2b). B_{max} values of 6.93 ± 1.04 and 7.38 ± 1.36 pmol/mg protein were recorded at 20 °C and 30 °C, respectively. Considering the analytical uncertainty, there was no significant difference. However, the [³H]NMS dissociation constants (K_D) differed significantly depending on incubation conditions: K_D values of 0.77 ± 0.11 nM and 5.06 ± 1.32 nM (n = 6) were calculated for 20 °C and 30 °C assay temperatures, respectively. During the complete study, the same batch of receptor membrane preparation aliquot for each assay was used to avoid inter-batch variations. A similar effect was observed when assays were performed in 10 mM HEPES buffer without $MgCl_2$.

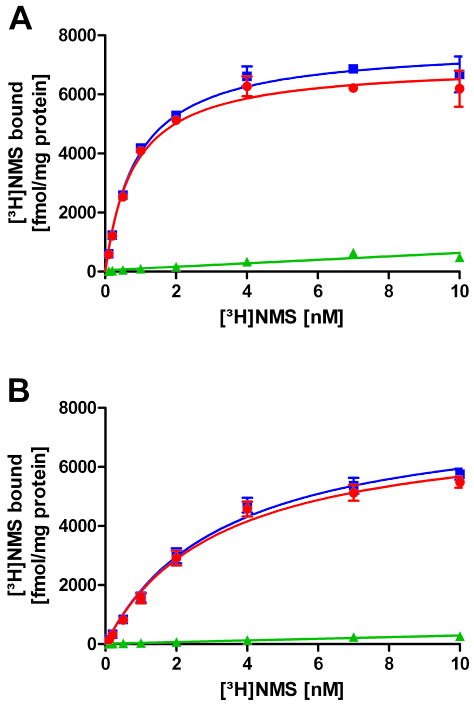

Figure 2. Saturation isotherms of [³H]NMS binding to hM_5 mAChR membrane fractions. (a) incubation temperature 20 °C. (b) incubation temperature 30 °C. Data are shown as means ± SEM (n = 3). Blue line and squares: total binding. Green line and triangles: non-specific binding. Red line and circles: specific binding.

Increasing of the incubation time to 4 h showed no significant altering of K_D values.

Preliminary studies in our laboratory reflected an even more drastic influence of the assay temperature on the affinity of the weak agonist [³H]oxotremorine M. At an incubation temperature of 30 C the affinity of [³H]oxotremorine M decreased by several orders of magnitude relative to 20 C (data not shown). This effect is probably related to the fact that [³H]oxotremorine M selectively labels only a fraction of the high-affinity receptors – a phenomenon which was observed for several mAChR agonists.[37] It appeared that at temperatures higher than 20 °C, the muscarinic acetylcholine receptor converted from a high affinity G-protein coupled state to a low-affinity G-protein uncoupled state. It was found that the optimum temperature for monitoring muscarinic receptor-G protein interactions in ligand binding assays was 16–20 C.[2]

Equilibrium and rate constants are known to be temperature dependent, with equilibrium binding of antagonists tending to be entropy driven.[38] With G-protein-coupled histamine H_3 receptors, increased temperature resulted in higher K_D values, but had no effect on B_{max} values.[39]

The K_D value of 0.77 ± 0.11 nM for [³H]NMS obtained in experiments with the hM_5 mAChR at the 20 °C incubation temperature was still slightly higher than K_D values previously reported (0.11-0.48 nM).[19–27] As already mentioned in the introduction, buffers, incubation temperature and time were different. According to the literature data, there was no evidence that higher temperatures led to higher K_D values.[23,24]

This is in contrast to the results achieved in this work. Rather, the different K_D values may depend on the selected buffer. The pH of HEPES buffer is more temperature-dependent than the pH of phosphate buffers, but HEPES is compatible with $MgCl_2$ whereas phosphate or citrate containing buffers may chelate cations, such as Mg^{2+}, added in millimolar concentrations. $MgCl_2$ may promote agonist binding to G-protein-coupled receptors by favouring the formation of the high-affinity agonist-receptor-G-protein complex.[40] To avoid variations within assay conditions, HEPES buffer was adjusted to pH 7.4 at the temperature used during incubation and washing. Another reason could be the lower ionic strength compared to the buffers cited in literature. Higher ionic strength seems to increase affinities.[41] In conclusion, it is evident that experimental factors (particularly the buffer composition) may influence ligand binding affinity.

Competition assays

Affinity constants measured for well-known probes of mAChRs and for the bispyridinium compounds, expressed as K_i [nM] and their negative logarithm (pK_i), appear in Table 1. The pK_i values obtained in this assay are in excellent agreement with previously recorded values in case of the antagonists 4-DAMP, pirenzepine, AQ-RA 741 and the agonist carbamoylcholine.[20,42–44] Figure 3 illustrates competition curves for selected agonists and antagonists. Affinity constants obtained under comparable assay conditions have not previously been published for scopolamine, oxotremorine M and 5-methylfurmethiodide. However, radioligand binding assays with recombinant hM_1-hM_4 mAChR yielded pK_i 8.7-9.5 for scopolamine,[45] pK_i 4.9-5.2 for oxotremorine M and pK_i 4.6–4.9 for 5-methylfurmethiodide.[46] In view of the fact that these ligands are not subtype-selective, the binding affinities of these compounds are in the same range for the M_1, M_2, M_3 and M_4 mAChRs.

Surprisingly, atropine displayed an approximately 10-fold weaker affinity (pK_i 8.5 ± 0.05) for the hM_5 mAChR than described in literature.[20,43] Similar results were obtained with other batches of atropine and [³H]NMS. Quality control by NMR spectroscopy and liquid chromatography-tandem mass spectrometry (LC-MS/MS) led to the conclusion that the original substance and the solutions were valid. Competition assays with hM_1 mAChR using identical solutions of atropine and [³H]NMS exhibited pK_i 8.6 ± 0.07 which was in agreement with cited values of 8.5-9.3.[45,47,48] In functional affinity estimates, obtained for antagonists against carbamoylcholine-stimulated [³H]inositol phosphates accumulation in CHO-K1 cells expressing human recombinant muscarinic M_5 receptors, pK_i values of 8.7 (atropine), 6.4 (pirenzepine), 6.1 (AQ-RA 741) and 8.6 (4-DAMP) were reported[49] which demonstrates an excellent agreement with the present data.

VU 0238429, described as a selective positive allosteric modulator of M_5 receptors,[50] revealed a weak activity (19 μM) at the M_5 orthosteric binding site.

The binding affinities of the tested bispyridinium compounds varied widely (Table 1). MB770, MB327, MB442, MB456 and MB583 exhibited K_i values of 1.4 μM, 3.3 μM, 6.6 μM, 8.6 μM and 10.8 μM, respectively. Maximum affinity was observed for MB770 which contains a phenyl substituent like other high-affinity ligands such as scopolamine, 4-DAMP, atropine and pirenzepine. Shortening the alkane linker from C3 (MB327) to C2 (MB583) in the 4-*tert*-butyl series resulted in lower affinity. With MB424, the only compound among those shown in Figure 1 without pharmacological benefit in soman-poisoned guinea pig diaphragm preparations,[29] no interaction with the orthosteric binding site was detected. Fluorination apparently causes loss of activity considering that the only difference between this compound and the good ligand MB327 is the presence of CF_3 groups rather than *tert*-butyl groups at the 4-position of the pyridine rings.

Figure 4 shows exemplary the displacement curve for MB770, the investigated bispyridinium compound with the highest

Table 1. Interaction of ligands with decreasing affinities at [³H]NMS binding sites of hM_5 mAChR. K_i is the equilibrium dissociation constant, pK_i is the negative logarithm of K_i. Data are means ± SEM (n = 3)

Ligand	K_i [nM]	pK_i	pK_i (cited)
Scopolamine	0.96 ± 0.09	9.0 ± 0.05	
4-DAMP	1.44 ± 0.16	8.8 ± 0.05	8.6-9.0 [20,42,43]
Atropine	3.15 ± 0.36	8.5 ± 0.05	9.1-9.7 [20,43]
Pirenzepine	102 ± 12	7.0 ± 0.05	6.2-7.1 [20,42,43]
AQ-RA 741	613 ± 120	6.2 ± 0.09	6.1 [42]
Oxotremorine M	4350 ± 649	5.4 ± 0.07	
5-Methylfurmethiodide	4470 ± 897	5.4 ± 0.09	
Carbamoylcholine	14900 ± 6220	4.8 ± 0.24	4.9 [44]
VU 0238429	19000 ± 7170	4.7 ± 0.21	
MB770	1440 ± 261	5.8 ± 0.09	
MB327	3300 ± 1120	5.5 ± 0.18	
MB442	6620 ± 1390	5.2 ± 0.10	
MB456	8550 ± 1800	5.1 ± 0.10	
MB583	10800 ± 2700	5.0 ± 0.12	
MB424	> 100000	< 4.0	

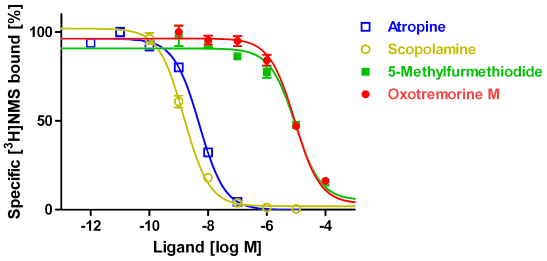

Figure 3. Competition binding curves with selected agonists and antagonists. Data are shown as means ± SEM (n = 6). Open symbols: antagonists. Filled symbols: agonists.

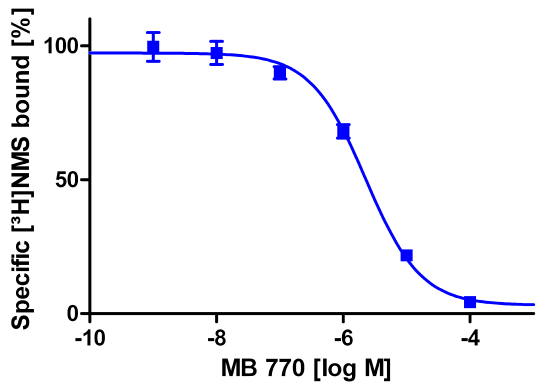

Figure 4. Competition binding curve of the bispyridinium compound MB770. Data are shown as means ± SEM (n = 6).

affinity. Compared to the [^{3}H]epibatidine binding sites of human α7 and *Torpedo* nicotinic acetylcholine receptors, the K_i values of MB770, MB442 and MB450 are approximately 10–100-fold lower.[51] Interestingly, an additional phenomenon was observed with the bispyridinium compound MB442. Specific binding of [^{3}H]NMS was correlated positively to the MB442 concentration and reached a maximum of 150% of total binding at approximately 100 nM MB442, and decreased at higher ligand concentrations. This could indicate a mixed mode of competitive and allosteric interactions[52] since such effects are known for other mAChR subtypes.[53,54] To confirm this hypothesis, further investigations, preferably functional assays,[55] will have to be performed.

Muscarinic receptors require that agonists bear a positive charge under physiological conditions: an aspartic acid residue on the receptor macromolecule provides the negative charge for ligand binding. The high-affinity probes tested – scopolamine, 4-DAMP, atropine, pirenzepine, AQ-RA 741, oxotremorine M, 5-methylfurmethiodide and carbamoylcholine – all possess this feature. The bispyridinium compounds have two positive charges. Whether M_5 mAChR interacted with both positive charges remains unclear, since no analogous monomeric compounds were investigated yet. However, the M_5 mAChR differed between the compounds to extents that depend on their degree of separation (compare binding data for C3 and C2 4-*tert*-butyl analogues, MB327 and MB583, with that for the C5 bispyridinium analogue MB442 lacking the 4-*tert*-butyl groups). There may be scope for optimisation of bispyridinium ligands for the hM_5 mAChR by exploring more fully the effect of different ring substituents and linker lengths on binding affinity.

Conclusion

The database on the binding of compounds to the human M_5 mAChR is small although this receptor is of relevance for the investigation of possible agents for the treatment of OP poisoning. Five of the six bispyridinium test compounds interacted with the orthosteric binding site, whereas one exhibited an atypical displacement curve that possibly indicated an allosteric or bitopic interaction with the hM_5 mAChR. Affinity of binding depended on two structural features in the bispyridinium compounds: the ring substituents and the length of the alkane linker separating the two rings. These preliminary results indicate that bispyridinium non-oximes interact with muscarinic as well as nicotinic receptors, and this interaction may contribute to their therapeutic efficacy in the treatment of OP poisoning *in vivo*.

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Affinities of bispyridinium non-oxime compounds to [^{3}H]epibatidine binding sites of *Torpedo californica* nicotinic acetylcholine receptors depend on linker length

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ABSTRACT

The toxicity of organophosphorus nerve agents or pesticides arises from accumulation of acetylcholine and overstimulation of both muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs) due to inhibition of acetylcholinesterase (AChE). Standard treatment by administration of atropine and oximes, e.g., obidoxime or pralidoxime, focuses on antagonism of mAChRs and reactivation of AChE, whereas nicotinic malfunction is not directly treated. An alternative approach would be to use nAChR active substances to counteract the effects of accumulated acetylcholine. Promising *in vitro* and *in vivo* results were obtained with the bispyridinium compounds SAD-128 (1,1′-oxydimethylene bis(4-*tert*-butylpyridinium) dichloride) and MB327 (1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium) di(iodide)), which were partly attributed to their interaction with nAChRs. In this study, a homologous series of unsubstituted and 4-*tert*-butyl-substituted bispyridinium compounds with different alkane linker lengths was investigated in competition binding experiments using [^{3}H]epibatidine as a reporter ligand. Additionally, the effect of the well-characterised MB327 on the [^{3}H]epibatidine equilibrium dissociation (K_D) constant in different buffers was determined. This study demonstrated that divalent cations increased the affinity of [^{3}H]epibatidine. Since quaternary ammonium molecules are known to inhibit AChE, the obtained affinity constants of the tested bispyridinium compounds were compared with the inhibition of human AChE. In competition experiments, bispyridinium derivatives of longer linker length displaced [^{3}H]epibatidine and inhibited AChE strongly. Bispyridinium compounds with short linkers, at most, have an allosteric interaction with the [^{3}H]epibatidine binding sites and barely inhibited AChE. In dependence on alkane linker length, the bispyridinium compounds seemed to interact at different binding sites. However, the exact binding sites of the bispyridinium compounds responsible for the positive pharmacological effects have still not been identified, making predictive drug design difficult.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are cation selective, pentameric ligand-gated ion channels that play important functional roles in cholinergic neurotransmission in both the peripheral (PNS) and central nervous system (CNS). As nAChRs are implicated in different CNS disorders (e.g., Alzheimer's and Parkinson's disease, schizophrenia, depression, tobacco addiction) and PNS disorders (e.g., congenital myasthenic syndrome, myasthenia gravis) [1,2], drugs that modulate nAChR function are becoming increasingly important [3–6]. Furthermore, nAChRs are interesting therapeutic targets for the treatment of intoxications, e.g., by organophosphorus compounds (OPCs) [7].

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) by phosphylation (phosphorylation and phosphonylation) of its active site serine [8,9]. The main physiological role of AChE is to hydrolyse the neurotransmitter acetylcholine to choline and acetate. Upon AChE inhibition, acetylcholine released from the nerve terminals accumulates in the synaptic cleft and over-stimulates both muscarinic (mAChRs) and nicotinic acetylcholine receptors (nAChRs). The disturbed cholinergic neurotransmission may ultimately result in central and peripheral respiratory failure, seizures, and other signs of cholinergic syndromes [10,11]. Standard treatment of OPC intoxication includes

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administration of atropine (a mAChR antagonist), an oxime (e.g., obidoxime or pralidoxime) to reactivate inhibited AChE, and a benzodiazepine (e.g., diazepam) to control seizures and convulsions [12,13]. In the case of nAChRs, functional recovery is only indirectly achieved by reactivation of AChE. AChE inhibited by certain OPCs is hardly reactivated by commonly used oxime AChE reactivators (tabun) or the enzyme–organophosphate complex undergoes a rapid dealkylation reaction (aging; soman), thus preventing reactivation by oximes [14].

Peripheral respiratory arrest due to neuromuscular block is a major problem and calls for approaches to recover neuromuscular transmission at respiratory muscles [15]. Consequently, muscle-type nAChRs are pharmacologically important targets for new therapeutic approaches to improve nerve agent-impaired neuromuscular transmission [16]. Previous studies with the bispyridinium non-oxime compound SAD-128 (1,1′-oxydimethylene bis(4-*tert*-butylpyridinium) dichloride) demonstrated its therapeutic effect against soman *in vitro* and *in vivo*, and partly attributed this to its interaction with nAChRs [17–21]. These observations 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 [22] explain the current interest in studying their interactions directly on muscle nAChRs.

Since suitable recombinant systems that stably express human muscle-type ($\alpha1\beta1\delta\varepsilon$) nAChRs in reasonable amounts were unavailable, investigations on receptor interactions were carried out with *Torpedo* electroplax membranes, a rich source of the $\alpha\beta\delta\gamma$ nAChR subtype localized at the neuromuscular junction [23]. *Torpedo* nAChRs show a high degree of homology with human muscle-type nAChRs [24]. For pharmacological characterisation of the [^{3}H]epibatidine binding sites on the *Torpedo* nAChR, binding protocols using Tris–HCl, HEPES or phosphate buffer were established [25,26]. In the present work, assays were performed in parallel using phosphate buffers differing in their concentrations of divalent cations. As part of this study [27,28], a series of bispyridinium non-oxime compounds bearing different lengths of *n*-alkyl linkers spanning the nitrogen atoms of the unsubstituted bispyridinium rings, and an analogous series of 4-*tert*-butyl-bispyridinium compounds, were tested on *Torpedo californica* plasma membrane preparations using a reasonable high-throughput assay performed on a custom-designed pipetting platform. Additionally, the interaction of bispyridinium analogues having an identical *n*-propan-1,3-diyl linker (C3), but different substitution pattern of methyl and ethyl groups on both pyridinium rings, with the [^{3}H]epibatidine binding sites was investigated. Since quaternary ammonium ligands are known to inhibit AChEs [29–31], the affinities obtained for all of the compounds tested in this study were compared to their inhibition potency at human erythrocyte AChE.

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, like filtermates and solid scintillators, were obtained from Perkin Elmer (Jügesheim, Germany). (±)-Epibatidine and α-bungarotoxin were purchased from Tocris (Bristol, UK) and pancuronium, carbamoylcholine, acetylthiocholine iodide and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent) from Sigma–Aldrich (Taufkirchen, Germany). The tested bispyridinium (MB) compounds (chemical structures see Tables 2–4) were synthesised at Dstl Porton Down, UK, and were ⩾98% pure by NMR spectroscopy and mass spectrometry [32]. Stock solutions of MB compounds, pancuronium, acetylcholine and carbamoylcholine were prepared in distilled water (1–10 mM), (±)-epibatidine in ethanol (1 mM), and α-bungarotoxin in 10% aqueous dimethyl sulfoxide (DMSO, 1 mM).

2.2. Preparation of nAChR enriched membrane fragments

Membranes from the frozen electric organ of *T. californica* were prepared as described before [33] with minor modifications at 4 °C. A threefold volume of extraction buffer (20 mM sodium phosphate buffer, 400 mM NaCl, 2 mM EDTA, freshly added 0.1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4) was added to frozen tissue. After thawing, the mixture was dispersed with an Ultra-Turrax at 13,500 rpm. The homogenate was centrifuged for 90 min at 27,000*g* (Beckman ultracentrifuge). The pellet was resuspended in wash buffer (extraction buffer without NaCl) and centrifuged for 60 min at 37,000*g*. After three washes, the suspension was centrifuged for 10 min at 1000*g*. The supernatant was mixed with ≈5-fold volume of pellet buffer (10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl, pH 7.4) and centrifuged for 30 min at 100,000*g*. The residue was suspended in a double volume of storage buffer (10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl, 300 mM sucrose, 0.5 mM EDTA, pH 7.4). Aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Sucrose gradient density centrifugation

To separate plasma membrane fractions from other subcellular membranes, subsequent purification by a modified sucrose gradient density centrifugation [34] was performed. The homogenate containing nAChR membrane fragments was added to an appropriate volume of 70% (w/w) sucrose solution (70% sucrose in 10 mM Tris–HCl buffer, pH 7.4) to reach a final sucrose concentration of 51%. After that, the membrane preparation was added to a discontinuous sucrose gradient (5.0 ml at 45%, 5.0 ml at 9%, w/w) in a 13.2 ml tube by underlayering, and centrifuged in a Beckman SE 41 TI rotor for 3 h at 100,000*g* and 4 °C. A total of approx. 3 ml fractions between the 9% and 45% layers were collected and transferred into a 36 ml tube, and then resuspended in buffer containing 10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl; pH 7.4. The protein suspension was centrifuged for 30 min at 100,000*g* and 4 °C. The supernatant was discarded and the pellet resuspended in two volumes of buffer composed of 10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl, 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. Aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C until use. The total protein concentration was determined by the bicinchoninic acid method [35,36], using bovine serum albumin as standard.

2.4. Radioligand binding

Radioligand binding experiments were performed according to a previously described [^{3}H]epibatidine assay method [27] with modifications. Pipetting and incubation were carried out with a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany). All radioligand experiments were conducted in 96-deep well plates in assay binding buffer (10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl, and different concentrations of divalent cations, 0–2 mM $CaCl_2$, 0–1 mM $MgCl_2$, pH 7,4). An aliquot of *Torpedo* membranes was rapidly thawed and diluted 200-fold with cold binding buffer, containing additional 1% bovine serum albumin. Total protein was approximately 10 μg per well

Table 1
Affinities of orthosteric ligands to [^{3}H]epibatidine binding sites of *Torpedo californica* membrane fractions using different assay buffers based on 10 mM sodium phosphate, 120 mM NaCl, 5 mM KCl, pH 7.4. Equilibrium dissociations constants (K_i) are expressed as the negative logarithm (pK_i). Inhibition of human AChE (IC_{50}) is expressed as the negative logarithm (pIC_{50}). All data are means ± SEM (n = 3).

Ligand	nAChR pK_i (buffer without $MgCl_2$, $CaCl_2$)	nAChR pK_i (buffer with 1 mM $MgCl_2$, 2 mM $CaCl_2$)	AChE pIC_{50}
(±)-Epibatidine	7.6 ± 0.1	7.9 ± 0.1	<3.7
Pancuronium	7.4 ± 0.1	7.8 ± 0.1	4.7 ± 0.0
α-Bungarotoxin	6.7 ± 0.1	6.4 ± 0.2	<3.7
Acetylcholine	*	6.1 ± 0.1	–
Carbamoylcholine	*	*	–

* Atypical competition curve.

(~1 μg Torpedo protein, ~9 μg BSA protein) and total volume was 250 μl in each well. The membrane suspension was stirred (200 rpm) at +4 °C. After incubation for 180 min at 20 °C, bound and free [^{3}H]epibatidine were separated by rapid vacuum filtration using a cell harvester (Perkin Elmer, Jügesheim, Germany) onto GF/B filter mates. The filtermates used for filtration were pre-soaked in 0.1% polyethylenimine [37] and dried at 40 °C overnight. The filter residue was rapidly washed five times with ice cold assay binding buffer. After 60 min drying at 50 °C, the membrane-containing filters were treated with a melt-on scintillator (MeltiLex B, Perkin Elmer-Wallac, Turku, Finland) for 4 min at 95 °C. Radioactivity was quantified using single photon counting on a MicroBeta scintillation counter (Perkin Elmer, Jügesheim, Germany) at ambient temperature. Concentrations of bound [^{3}H]epibatidine were calculated with a calibration curve (linear regression). To obtain the calibration curve, aliquots of radiolabelled marker used in the assay were applied to the filter mate before melting of the solid scintillator. In all experiments, the total ligand binding never exceeded more than 10% of the added ligands to limit complications associated with depletion of the free radioligand concentration [38,39].

2.5. [^{3}H]Epibatidine saturation binding

Binding was performed with assay concentrations of 1, 2, 5, 10, 20, 40, 70 and 100 nM [^{3}H]epibatidine to obtain saturation binding curves. Non specific binding was determined in the presence of 10 μM (±)-epibatidine. Aliquots of protein suspension (~10 μg/well total protein, ~1 μg/well *Torpedo* protein) were incubated at 20 °C with gentle agitation (~300 rpm) for 180 min. Binding data were calculated from triplicates.

2.6. Competition assays

To obtain the binding affinity of unlabelled compounds to [^{3}H]epibatidine binding sites, competition experiments were performed. The concentration of [^{3}H]epibatidine was 3 nM in the assay. Total binding was determined without competitor and non-specific binding in the presence of 10 μM unlabelled (±)-epibatidine in the assay. [^{3}H]Epibatidine was incubated in the presence of six indicated concentrations of the unlabelled compounds and protein suspension (~10 μg/well total protein, 1 μg/well *Torpedo* protein) at 20 °C with gentle agitation for 180 min. The estimation of K_i values included three independent experiments, each performed in six replicates per concentration.

2.7. Data analysis

Specific binding was defined as the difference between total binding and non-specific binding. The concentration of a competing test compound that inhibited 50% of specific binding (IC_{50}) was calculated with nonlinear regression (Prism 5.0, GraphPad Software, San Diego, CA, USA) for sigmoidal dose–response curves obtained in competitive binding experiments (One site – Fit K_i). The top and bottom of the sigmoidal curve were constrained to values obtained for total binding (without competitor, top) or non-specific binding (in the presence of 10 μM epibatidine, bottom). K_i values were determined according to the Cheng & Prusoff equation [40]. Values for the equilibrium dissociation constant (K_D) and the maximum density of binding sites (B_{max}) were calculated from binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 5.0). Results are expressed as means ± SEM (n = 3).

2.8. AChE activity

Hemoglobin-free human erythrocyte ghosts, used as the AChE source, were prepared as previously described [41] with minor modifications [42]. For the determination of AChE activity, a modified colorimetric Ellman assay [43,44], using acetylthiocholine (0.45 mM) as substrate and DTNB (0.3 mM) as chromogen, was performed in 0.1 M phosphate buffer (pH 7.4) at 37 °C.

2.9. AChE inhibition assay

AChE inhibition was investigated as previously described [27]. In brief, inhibitory potency of the bispyridinium compounds was determined by incubation of AChE with 10^{-6}–10^{-3} M test compound. The IC_{50} was calculated from semi-logarithmic plots of bispyridinium compound concentration versus AChE activity.

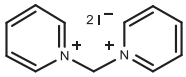
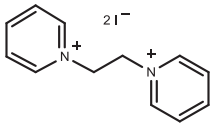
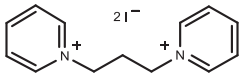
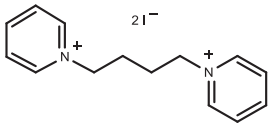
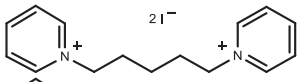
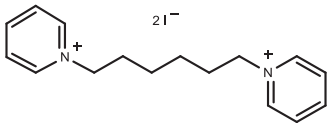
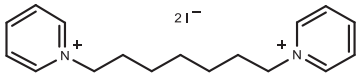
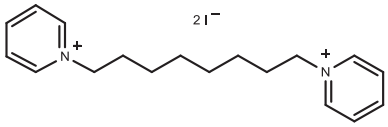
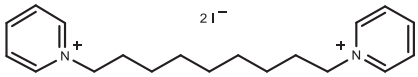
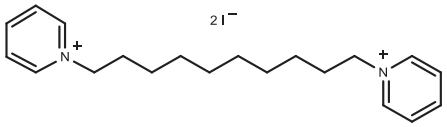
3. Results and discussion

3.1. [^{3}H]Epibatidine binding

It should be noted that only the total number of binding sites without differentiation between high and low affinity binding sites was calculated. During the present study, the same batch of receptor plasma membrane preparation (B_{Max} 75.9 ± 3.2 pmol/mg protein; n = 6) for each assay was used to avoid inter-batch variations. The comparison of the [^{3}H]epibatidine equilibrium dissociation constants (K_D) revealed a significant influence of buffer composition on [^{3}H]epibatidine K_D, especially with regard to divalent cations like calcium and magnesium (Fig. 1).

In absence of the divalent cations $CaCl_2$ and $MgCl_2$, the resulted saturation isotherms level off, causing increasing K_D values. In this case, valid calculations of B_{Max} and K_D were not successful at maximal [^{3}H]epibatidine concentrations of 100 nM (Fig. 1). In preliminary experiments, which were performed with [^{3}H]epibatidine concentrations up to 500 nM, the use of assay buffers without divalent cations resulted in K_D = 28.6 ± 3.7 nM. When the buffer contains 1 mM $MgCl_2$ but no $CaCl_2$ K_D = 17.6 ± 3.0 nM was achieved. In the case of G protein-coupled receptors (GPCRs), Mg^{2+} is known to promote agonist binding by favouring the formation of the high-affinity agonist-GPCR complex [45]. However, whether Mg^{2+} induces a high affinity conformation of the ion

Table 2
Affinities of unsubstituted bispyridinium compounds to [^{3}H]epibatidine binding sites of *Torpedo californica* membrane fractions using different assay buffers based on 10 mM sodium phosphate, 120 mM NaCl, 5 mM KCl, pH 7.4. Equilibrium dissociations constants (K_i) are expressed as the negative logarithm (pK_i). Inhibition of human AChE (IC_{50}) is expressed as the negative logarithm (pIC_{50}). All data are means ± SEM (n = 3).

Ligand	Structure	nAChR pK_i (buffer without $CaCl_2$, $MgCl_2$)	nAChR pK_i (buffer with 1 mM $MgCl_2$, 2 mM $CaCl_2$)	AChE pIC_{50}
MB775		<4.0	<4.0	<3.0
MB520		<4.0	<4.0	<3.0
MB408		4.7 ± 0.1	4.4 ± 0.2	<3.0
MB444		*	*	<3.0
MB442		4.7 ± 0.1	4.4 ± 0.2	<3.0 [27]
MB776		*	4.6 ± 0.1	4.1 ± 0.0
MB777		5.0 ± 0.1	5.1 ± 0.1	4.8 ± 0.0
MB505		5.2 ± 0.0	5.1 ± 0.1	5.8 ± 0.2
MB778		5.5 ± 0.1	5.6 ± 0.0	6.5 ± 0.0
MB779		5.7 ± 0.0	5.8 ± 0.0	6.7 ± 0.0

* Atypical competition curve.

receptor channel or has a stabilizing influence on the membrane [46,47] and indirectly on the receptor, remains speculative. Previous studies supported the positive allosteric effect of Ca^{2+} [48], which was only barely mimicked by Mg^{2+} [49].

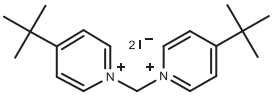
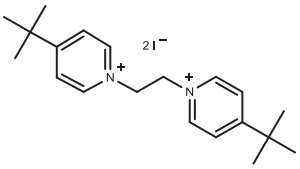
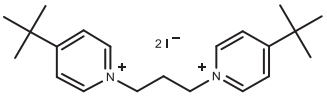
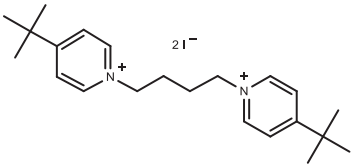
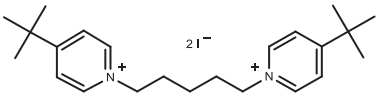
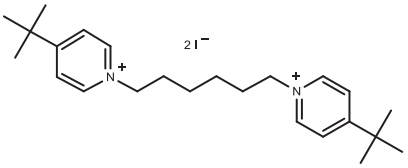
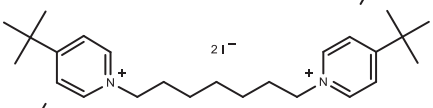
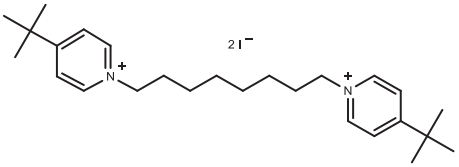
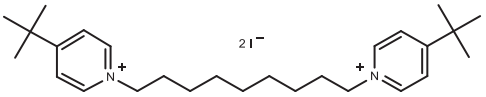
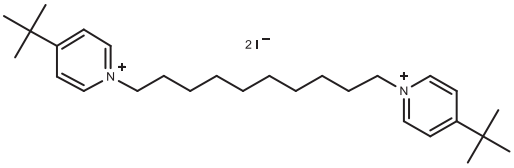
When the binding buffer contained 1 mM $MgCl_2$ and 2 mM $CaCl_2$, the [^{3}H]epibatidine affinity increased significantly in comparison to data obtained with buffers without divalent cations (K_D = 10.4 ± 3.2 nM). Further increases of Mg^{2+}/Ca^{2+} concentrations were not compatible as buffer components precipitated. In the presence of both divalent cations, nAChRs seemed to exist in a high affinity conformational state, whereas in the absence of calcium and magnesium ions, receptor conformations were observed that were associated with low [^{3}H]epibatidine affinities. For α7 nAChR, modulation by divalent cations involves conformational changes in the extracellular domain [50]. Calcium ions, but not magnesium ions, have been shown to potentiate nAChR-mediated responses in an allosteric fashion [49,51]. Possibly related to calcium binding sites located in the extracellular domain distinct from the orthosteric binding sites [52], the induced conformational changes resulted in higher affinities to agonists like epibatidine. However, the data from this study allow no distinction between the effect attributed to the occupying of binding sites by calcium or magnesium ions, or to other effects, e.g., membrane stabilizing effects caused by increased total concentrations of divalent cations.

In summary, the presence of divalent cations led to higher [^{3}H]epibatidine affinities.

3.2. Orthosteric ligands

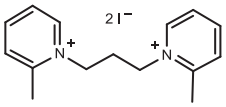
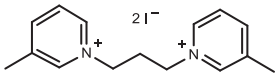
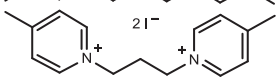
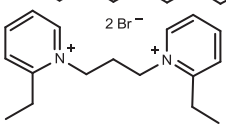
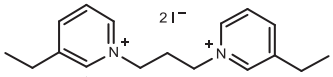
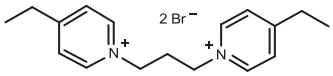
Given the assumption that divalent cations induced a high-affinity conformation receptor state, this may also influence the affinity of competitors, and therefore buffers containing 1 mM

Table 3
Affinities of 4-*tert*-butyl-substituted bispyridinium compounds to [^{3}H]epibatidine binding sites of *Torpedo californica* membrane fractions using different assay buffers based on 10 mM sodium phosphate, 120 mM NaCl, 5 mM KCl, pH 7.4. Equilibrium dissociations constants (K_i) are expressed as the negative logarithm (pK_i). Inhibition of human AChE (IC_{50}) is expressed as the negative logarithm (pIC_{50}). All data are means ± SEM (n = 3). Squared bracket: interference with allosteric effects.

Ligand	Structure	nAChR pK_i (buffer without $CaCl_2$, $MgCl_2$)	nAChR pK_i (buffer with 1 mM $MgCl_2$, 2 mM $CaCl_2$)	AChE pIC_{50}
MB780		4.6 ± 0.4	<4.0	<3.0
MB583		*	<4.0	3.2 ± 0.0 [27]
MB327		*	<4.0	3.2 ± 0.0 [27]
MB781		<4.0	[4.5 ± 0.1]	4.5 ± 0.2
MB782		4.5 ± 0.2	[4.9 ± 0.1]	5.4 ± 0.1
MB783		<4.0	[5.4 ± 0.1]	6.2 ± 0.1
MB784		<4.0	5.5 ± 0.1	6.2 ± 0.1
MB785		4.9 ± 0.1	5.2 ± 0.1	6.5 ± 0.0
MB582		*	*	6.7 ± 0.0
MB786		5.1 ± 0.1	5.5 ± 0.1	7.0 ± 0.0

* Atypical competition curve.

Table 4
Affinities of methyl and ethyl substituted bispyridinium compounds to [^{3}H]epibatidine binding sites of *Torpedo californica* membrane fractions using different assay buffers based on 10 mM sodium phosphate, 120 mM NaCl, 5 mM KCl, pH 7.4. Equilibrium dissociations constants (K_i) are expressed as the negative logarithm (pK_i). Inhibition of human AChE (IC_{50}) is expressed as negative logarithm (pIC_{50}). All data are means ± SEM (n = 3).

Ligand	Structure	nAChR pK_i (buffer without $CaCl_2$)	nAChR pK_i (buffer with 1 mM $MgCl_2$, 2 mM $CaCl_2$)	AChE pIC_{50}
MB420		<4.0	<4.0	<3.0
MB419		<4.0	<4.0	3.5 ± 0.0
MB414		<4.0	<4.0	3.2 ± 0.0
MB455		4.5 ± 0.2	<4.0	3.4 ± 0.0
MB421		<4.0	<4.0	3.6 ± 0.0
MB454		<4.0	*	3.5 ± 0.0

* Atypical competition curve.

$MgCl_2$ and 2 mM $CaCl_2$, and buffers free of divalent cations, were used in parallel in competitive binding studies (Table 1). In addition, inhibition potency on human erythrocyte AChE was investigated (Table 1). Although the ligands used in this study address nAChR orthosteric binding sites, their occupation of these binding sites is not identical, depending on formation of different hydrogen bonds and cation-Π interactions [52–58].

According to the [^{3}H]epibatidine dissociation constants (K_D), affinity of (±)epibatidine was affected by the buffer composition: in absence of divalent cations, pK_i 7.6 ± 0.1 was recorded. The affinity constant pK_i 7.9 ± 0.1 in the presence of 1 mM $MgCl_2$ and 2 mM $CaCl_2$ was in close agreement with those from (±)epibatidine affinity experiments performed on affinity-purified and lipid-reconstituted *Torpedo* membranes (pK_i = 8.0 ± 0.1) [26]. The non-depolarizing muscle relaxant pancuronium [59,60] showed increasing affinity to [^{3}H]epibatidine binding sites when divalent cations were present, whereas the effects on α-bungarotoxin affinity were contrary. α-Bungarotoxin is described as a potent antagonist that binds with high affinity to [^{125}I]α-bungarotoxin binding sites of *Torpedo* nAChRs (pK_i = 9.2–9.6) [61]. Affinity data are difficult to compare as epibatidine and α-bungarotoxin bind not in the same way to orthosteric binding sites [53]. Furthermore, not only is the radioactive labelled reporter ligand different from previous experimental conditions, differences in the membrane preparation may also influence binding affinity. Non-purified plasma membrane preparations also contain endoplasmic reticulum (ER) membranes; it was postulated that after subunit folding and assembly events that occur in the ER, nAChRs cluster during synaptogenesis at the neuromuscular junction [62]. α-Bungarotoxin seems to bind even at the non-mature hetero-oligomers of nAChRs, which are present in the ER. Here, plasma membrane preparation by sucrose density centrifugation is expected to result in a membrane preparation deficient in ER nAChRs. Hence, it may be assumed that binding of α-bungarotoxin to a heteromerically diverse subunit population is unlikely to occur. Consequently, in the experiments purified plasma membrane fractions were used.

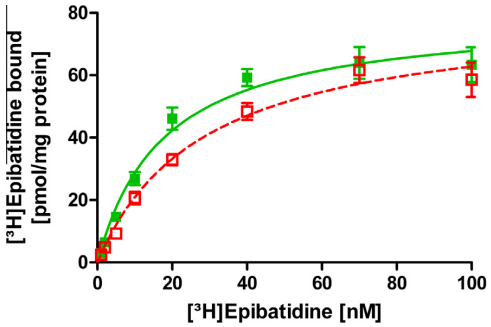


Fig. 1. Selected saturation isotherms of [^{3}H]epibatidine binding to *Torpedo californica* membrane fractions. Data are shown as means ± SD (n = 3). Green solid line, triangles: buffer with divalent cations (1 mM $MgCl_2$, 2 mM $CaCl_2$), red dashed line, open squares: buffer without divalent cations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Interestingly, acetylcholine and carbamoylcholine exhibited curve artefacts in the absence of divalent cations, while in presence of 1 mM $MgCl_2$ and 2 mM $CaCl_2$, [^{3}H]epibatidine displacement was seen more clearly (Fig. 2). Competition of carbamoylcholine interfered probably with allosteric effects, resulting in a curve which indicated an enhancement of [^{3}H]epibatidine binding at approx. 1 μM carbamoylcholine. Interestingly, assays performed with plasma membrane fractions from GH_4C_1 cells stably expressed human α7 nAChRs exhibited regular competition curves, even if buffers without divalent cations were used (Fig. 2). The endogenous agonist acetylcholine interacted with [^{3}H]epibatidine binding sites at nanomolar concentrations (K_i ~800 nM). This result needs careful interpretation because acetylcholine underwent a non-enzymatic hydrolysis to acetate and choline during incubation. Plasma

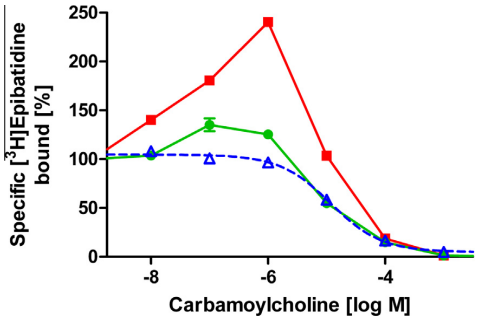


Fig. 2. Selected competition assays for the nAChR agonist carbamoylcholine. Red solid line, squares: buffer without divalent cations, green solid line and circles: buffer with divalent cations (1 mM $MgCl_2$, 2 mM $CaCl_2$), blue dashed line and open triangles: buffer without divalent cations and human α7 nAChR subtype. Data are means ± SD ($n = 6$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

membrane preparations used in this assay had ~400 mU AChE activity/mg protein measured at 20 °C. It should be noted that no AChE blocker was used, because possible interactions with nAChR could not excluded. Enzymatic and non-enzymatic hydrolysis therefore decreased acetylcholine concentrations during competition assays and resulted in an underestimation of the acetylcholine affinity.

The interaction of orthosteric nAChR ligands with human AChE was tested since these compounds bear quaternary ammonium structures or protonated nitrogen under the applied experimental conditions. Therefore, the nAChR agonist (±)epibatidine and nAChR antagonists α-bungarotoxin and pancuronium were tested for their ability to affect human AChE. (±)Epibatidine and α-bungarotoxin did not affect human AChE at concentrations >200 μM ($pIC_{50} < 3.7$, Table 1). In contrast, the nAChR blocker pancuronium inhibited AChE in low micromolar concentrations (IC_{50} ~20 μM). The inhibition was reversible and concentration dependent (Fig. 3).

3.3. Unsubstituted bispyridinium compounds

Testing of a homologous series of unsubstituted bispyridinium compounds with C1–C10 alkane linkers resulted in different effects on the *T. californica* nAChR. In the absence of divalent cations, bispyridinium compounds including C7–C10 linkers displaced [^{3}H]epibatidine from its binding site, resulting in regular competition curves (Fig. 4). Here the linker length was positively correlated to the affinity for [^{3}H]epibatidine binding sites, and this was observed in buffers both with and without calcium (Table 2). In contrast, the bispyridinium compound MB776 with an C6 linker did not show a typical displacement of [^{3}H]epibatidine from its binding sites in buffer without calcium and magnesium.

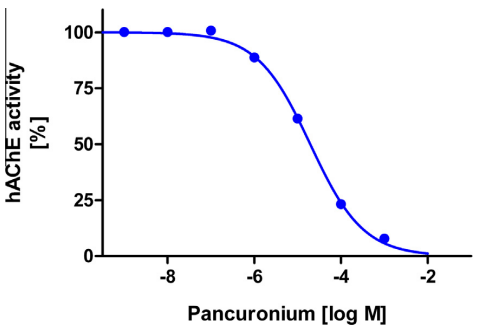


Fig. 3. Inhibition of human AChE by pancuronium. Data are shown as means ± SD ($n = 4$).

The observed effect is consistent with previously described affinities to [^{3}H]nicotine binding sites in rat brain homogenates, mainly containing α4β2 and α7 receptor subtypes [63]. Bispyridinium analogues including ones with long *n*-alkyl linkers, such as *N*,*N*′-decane-1,10-diyl-bispyridinium diiodide (bPDI (C10), exhibited micromolar affinities in the [^{3}H]nicotine binding assay ($pK_i = 4.7$) [63]. Related to the well-established pharmacological profile of the bis-trimethylammonium alkanes (e.g., hexamethonium, decamethonium), quaternary compounds with a high affinity to [^{3}H]nicotine binding sites inhibited nicotine evoked $^{86}Rb^+$ efflux from rat thalamic synaptosomes [63]. Although these results were obtained with different receptor membrane preparations and radioactive tracers, similar effects were recorded in the present study. The bispyridinium compound MB779 (C10) demonstrated the highest affinity ($pK_i = 5.8$, corresponding to K_i ~1.6 μM). Probably, the bispyridinium compounds that exhibited affinity for the [^{3}H]epibatidine binding sites acted also as antagonists. Experiments based on indirect electric field stimulation using rat diaphragm confirmed that recovery of soman blocked neuromuscular function by non-oxime bispyridinium compounds depends on their linker length [64]. However, further investigations are necessary to confirm this hypothesis.

The biological activity of aliphatic bisquaternary ammonium compounds depends on the linker length and on its rigidity. For example, the well known AChE inhibitor decamethonium [29] acts as a partial agonist [65], whereas hexamethonium and dodecamethonium inhibit muscle nicotinic responses [66]. Long bisquaternary dicholine agonists like succinylcholine (18.7 Å between quarternary nitrogens) appeared to bind to multiple sites on muscle-type nAChRs [67]. Further it was shown that the bisquaternary compound 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) acted as an allosteric effector and an open channel blocker of muscle-type nAChRs [68].

Since only [^{3}H]epibatidine binding sites are considered here, it cannot be excluded that the bispyridinium compounds tested in this study may have occupied further binding sites, perhaps acting as bitopic ligands [69]. However, since no functional tests were performed, their intrinsic effect, i.e., agonism or antagonism of nAChRs, could not be evaluated clearly by the [^{3}H]epibatidine affinity experiments only.

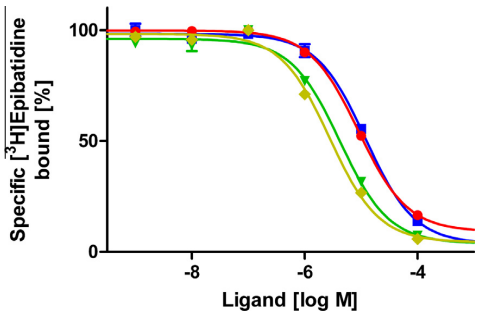


Fig. 4. Selected competition assays for the unsubstituted bispyridinium compounds: MB777 (C7, blue squares), MB505 (C8, red circles), MB778 (C9, green triangles) and MB779 (C10, yellow rhombi) performed in binding buffer without divalent cations. Total binding was determined without ligand and non-specific binding in the presence of 10 μM (±)epibatidine. Data are means ± SD ($n = 6$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Inhibition of human erythrocyte AChE depended strongly on the linker length. Compounds with C1–C5 linkers did not inhibit AChE (IC_{50} > 1 mM), whereas the IC_{50} value of MB776 (C6 linker) fell rapidly to ~80 μM (pIC_{50} = 4.1 ± 0.0; Table 2). If the distance between the pyridinium rings was extended (C9, C10), AChE was inhibited at nanomolar concentrations (pIC_{50} 6.5–6.7, corresponding to IC_{50} ~200–300 nM). Compared to the affinity constants for the [^{3}H]epibatidine binding sites, clear correlations could be found: compounds that had a higher affinity to [^{3}H]epibatidine binding sites (pK_i > 5, corresponding to <10 μM) inhibited AChE relatively strongly. For drug development, strong inhibition of AChE could be a disadvantage.

3.4. 4-Tert-butyl-substituted bispyridinium compounds

In general, the binding of 4-*tert*-butyl bispyridinium analogues to [^{3}H]epibatidine binding sites and the inhibition of AChE was comparable to unsubstituted bispyridinium compounds. However, the affinity profiles of 4-*tert*-butyl bispyridinium analogues were heterogeneous with enhancement of [^{3}H]epibatidine binding apparent in some cases (Table 3).

When linker length was C8 and longer, [^{3}H]epibatidine was displaced in a simple competitive manner, except with MB582 (C9 linker), which induced a ~170% enhancement of [^{3}H]epibatidine binding at a range of 0.1–1 μM (Fig. 5 and Table 3), regardless of assay buffer composition. Probably, the binding of MB582 to its locus provokes a local conformational change that allows [^{3}H]epibatidine to fit more readily within its binding domain, as observed with other ligands [69], assuming that the distinct binding sites are allosterically linked; photoaffinity labelling experiments have detected allosterically linked binding sites for noncompetitive and competitive antagonists [70]. Further experiments are necessary to explain these effects.

In the case of the normally competitive compounds, MB785 (C8) and MB786 (C10), a slight increase of affinity was observed if the buffers were supplemented with 1 mM $MgCl_2$ and 2 mM $CaCl_2$. Notably, MB784 (C7) did not compete with the [^{3}H]epibatidine in the absence of divalent cations, whereas in the presence of divalent cations micromolar affinities were observed.

In the case of 4-*tert*-butyl-bispyridinium compounds having a C5–C6 spacer, two different effects overlapped: firstly, a weak increase of affinity for [^{3}H]epibatidine up to approximately 1 μM of the competing compound was observed and secondly, the [^{3}H]epibatidine binding was displaced by higher competitor concentrations. These phenomena may suggest an overlaying of orthosteric and allosteric effects, characterised by occupation of binding sites of the agonist [^{3}H]epibatidine and an indirect influence of [^{3}H]epibatidine binding (in this case, enhancement). For MB327 (C3), displacement of [^{3}H]epibatidine was very weak (>100 μM). Lack of divalent cations (especially calcium) induced stronger (~130%) enhancement of [^{3}H]epibatidine binding (Fig. 6). MB327 had been well characterised in several pharmacological investigations *in vitro* (muscle force in human intercostal muscle strips [71] and diaphragms of different species [64,72]) and *in vivo* [73]. Further, functional assays using identical plasma membrane preparations generated results concerning sodium conductance of activated nAChRs. During measurements of transient cholinergic currents of *T. californica* nAChRs using a cell-free electrophysiological method based on solid supported membranes in the presence of MB327, no carbamoylcholine-induced desensitisation was observed [74].

When 4-*tert*-butyl-bispyridinium compounds having long linkers (C8–C10) were applied on soman poisoned rat diaphragm, no recovery was observed [64]. That suggests that these compounds are nAChR antagonists, like their unsubstituted counterparts. However, antagonism or other additional effects could not be confirmed, since no results of functional investigations directly on nAChR have been published so far.

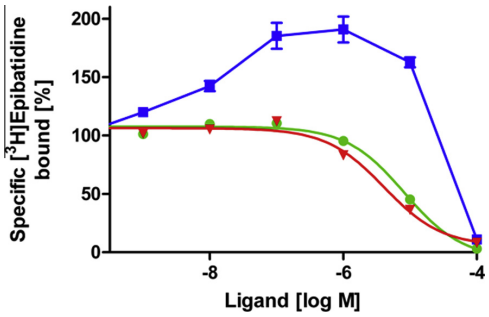


Fig. 5. Selected competition assays for the 4-*tert*-butyl substituted bispyridinium compounds MB785 (C8, green circles), MB582 (C9, blue squares) and MB786 (red triangles). Assays were performed in binding buffer with divalent cations. Total binding was determined without ligand and non-specific binding in the presence of 10 μM (±)epibatidine. Data are means ± SD (n = 6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Analogous to the results obtained for the unsubstituted bispyridinium series, 4-*tert*-butyl-bispyridinium derivatives with extended linkers (C6–C10) inhibited human AChE (Table 3) at low concentrations (pIC_{50} 6.2–7.0, corresponding to IC_{50} ~100–600 nM). Comparable to the unsubstituted bispyridinium compounds, the linker length correlated with the inhibitory potency towards AChE. Compared to affinity constants for [^{3}H]epibatidine binding sites, almost all compounds with high pIC_{50}, > 6 (or low IC_{50} values, <1 μM) generated relatively high pK_i values (>5.0). However, the correlation was less pronounced in comparison to the unsubstituted bispyridinium compounds.

3.5. Methyl- and ethyl-substituted bispyridinium compounds

The six compounds tested (Table 4) shared hardly affinity (pK_i < 4.0, corresponding to K_i > 100 μM) to [^{3}H]epibatidine binding sites irrespective of the buffer composition used, although the 2-ethyl substituted compound MB455 displaced [^{3}H]epibatidine quite weakly, In the presence of divalent cations, the 4-ethyl analogue MB454 demonstrated a slight (~120%) enhancement of [^{3}H]epibatidine binding. As mentioned earlier, the effects observed in the [^{3}H]epibatidine affinity experiments allow no conclusions to

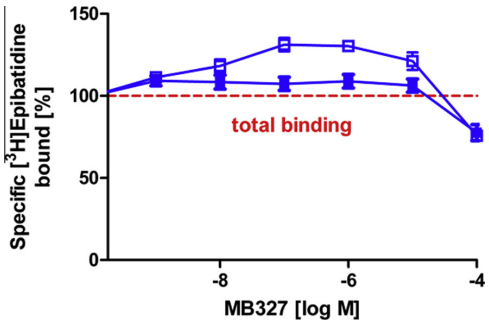


Fig. 6. Selected competition assays for the 4-*tert*-butyl bispyridinium compound MB327 (C3) performed in binding buffer without divalent cations (solid squares) and in presence of 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (open squares). Total binding was determined in absence of MB327 and non-specific binding in the presence of 10 μM (±)epibatidine. Data are means ± SD (n = 6).

be made concerning intrinsic effects on muscle-type nAChRs. Functional measurements are essential to elucidate the effects on nAChRs directly.

Regarding inhibition potency of human AChE (Table 4), the 2-methyl bispyridinium derivative (MB420) had no effect ($pIC_{50} < 3.0$; corresponding to $IC_{50} > 1$ mM); however, methyl bispyridinium compounds substituted in the 3- and 4-positions (MB419 and MB414) inhibited AChE in micromolar concentrations ($pIC_{50} = 3.9 \pm 0.0$ and 3.2 ± 0.0, corresponding to IC_{50} ~300 and IC_{50} ~600, respectively). In the ethyl-substituted series, the position of the side chain did not appear to affect the weak inhibitory efficacy on human AChE. Compared to unsubstituted and 4-*tert*-butyl substituted C3 analogues (Tables 2 and 3), addition of methyl or ethyl groups to both pyridinium units generated similar effects with respect to competition of [^{3}H]epibatidine binding and inhibition of human AChE.

4. Conclusions

These data suggest that some of the compounds can bind to [^{3}H]epibatidine binding sites of muscle-type nAChRs and that binding affinity is somewhat dependent on their linker length. [^{3}H]Epibatidine binding to *T. californica* nAChRs was inhibited by bispyridinium compounds with long alkyl linkers. Additionally, these compounds exhibited a remarkable inhibitory potency towards human AChE. Finally, literature data indicate an antagonistic effect of bispyridinium compounds having long alkane linkers at nAChRs. However, this hypothesis has to be confirmed by functional measurements.

Further, it was demonstrated that the presence of divalent cations enhanced [^{3}H]epibatidine binding. However, it remains unclear which mechanism is responsible for this effect. It should be noted that only binding sites addressed by [^{3}H]epibatidine were investigated in this study. Probably other binding sites were occupied, partly allosterically linked with the [^{3}H]epibatidine binding sites, expressed by an enhancement of radioisotope labelled tracer binding in some cases. Experiments based on other reporter ligands are necessary to locate binding sites that are addressed by small-sized bispyridinium compounds (C2–C5). Additionally, functional assays must be performed to clarify the intrinsic effect on nAChRs. Lastly, substances that include different pharmacophores have to be investigated, enabling more successful predictive drug design and the generation of new nicotinic drugs for conditions caused by OP intoxications, where they could offer therapeutic benefits.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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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.

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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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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 $\alpha\beta\delta g$ 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.

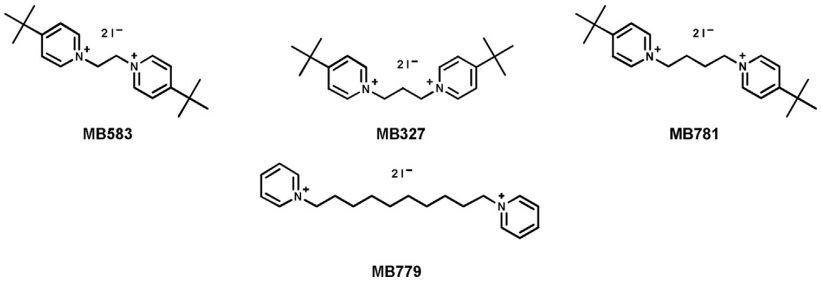

Fig. 1. Selected non-oxime bispyridinium compounds tested in the present study.

2.3. Preparation of protein vesicles

The plasma membrane fragments were treated by two freeze–thaw cycles and homogenisation using an extruder device (Polar Lipids, Alabaster, AL, USA) to achieve consistent vesicles. Afterwards, the total protein concentration of an aliquot was determined by the bicinchoninic acid method (Smith et al., 1985; Redinbaugh and Turley, 1986), using bovine serum albumin as standard. The remaining protein suspension was stored at −150 °C until use.

2.4. Photon correlation spectroscopy (PCS)

The parameter average particle size (Z_{avg}), polydispersity index (PI), electrophoric mobility (U) and zeta potential (ζ) were measured in combination using a photon correlation spectrometer (Horiba, distributed by Retsch Technology, Haan, Germany). The protein suspension was treated by sonication (1 burst, 50 W, 30 kHz, 1 mm tip Diameter, intensity 30%, cycle 0.5) and filtration (0.8 μM cellulose-acetate) and then carefully diluted with buffer (100 mM NMG-H_2SO_4, 30 mM Tris, 3 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 by HCl) to reach the final concentration of 10 μg/ml. Intense mixing by shaking or vortexing that might alter radically vesicle properties was avoided. The diluted protein suspension was pipetted into fused silica cuvettes, followed by the measurement of Z_{avg} and PI. The reading was performed at a 90° angle with respect to the incident beam. For calibration polystyrene standards were used with the particle sizes 742 ± 11 nm and 107 ± 4 nm. Z_{avg} and PI were calculated by the software "Horiba SZ-100" based on autocorrelation.

The determination of U and ζ was carried out by laser Doppler electrophoresis using the same instrument. Therefore, 100 μl of the diluted protein suspension was placed into the electrophoretic cells, where a potential of ±1.5 mV was induced. 10 single measurements were performed in the mode "organic sample" and the refractive index for aqueous milieu (n = 1.333). U and ζ were calculated by the software, using the Hückel equation.

2.5. Radioligand binding

[^{3}H]Epibatidine saturation experiments were performed as previously described (Niessen et al., 2013). In brief, pipetting and incubation were carried out with a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany). All radioligand experiments were conducted in 96-well plates in assay binding buffer (10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl; pH 7.4) using 1–100 nM [^{3}H]epibatidine for total binding and 10 μM (±)-epibatidine for non-specific binding. Specific binding was defined as the difference between total binding and non-specific binding. In all experiments, the total ligand binding never exceeded more than 10% of the added ligands to limit complications associated with depletion of the free radioligand concentration (Rovati, 1993; Carter et al., 2007). The maximum density of binding sites (B_{max}) was calculated from binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (GraphPad Prism 5.0). Results were expressed as means ± SEM (n = 3).

2.6. Sensor chip preparation

The preparation of the sensor chips carrying the electrode were performed as previously described (Schulz et al., 2008) with the following modifications. Briefly, the sensor chips (Nanion, Munich, Germany) were pre-treated by incubation with a special cleaning agent (Helmanex® II, Müllheim, Germany, 4% in water) for 10 min, drying under nitrogen gas, incubation in permonosulfuric acid (freshly prepared by addition of 1 part H_2O_2 (30%) and 3 parts H_2SO_4 (98%)) for 2 min, and finally drying in a gentle stream of nitrogen gas after rinsing with water. For storage the sensor chips were kept in nitrogen atmosphere and shielded from UV-light. After cleaning, the sensor chips were regenerated by 1 h incubation in an octadecanethiol solution (1 mM in isopropyl alcohol). One day before the measurements, 1.2 μl of the lipid–emulsifier mixture, containing 15 mg/ml diphytanoyl-phosphatidylcholine and 250 μg/ml octadylamine, were carefully applied onto the sensor chip without contact with the octadecanethiol solution. The components of the solid supported membrane (SSM) were prepared as follows: 375 μl of diphytanoyl-phosphatidylcholine (20 mg/ml in chloroform) and 25 μl of octadecylamine (5 mg/ml in chloroform) were added to a glass vial and the solvent was removed by a gentle stream of nitrogen gas. The residue was reconstituted by 500 μl n-decane and stored at −20 °C until use. Immediately after the lipid-emulsifier mixture was added, 60 μl of buffer C (100 mM NMG-H_2SO_4, 30 mM Tris, 3 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 by HCl) were added, the sensor sealed by a chemically inert label (ToughSpot®, Sigma-Aldrich, Taufkirchen, Germany) and stored at 4–8 °C for 1 h. The sensor was placed in the SURFE^{2}R 500 workstation (Nanion, Munich, Germany) und washed with rinsing buffer resulting in the spontaneous formation of the SSM. To check the quality of the SSM, capacitance and conductance were measured. The SSM was formed correctly, when the capacitance measured ~3 nF and the conductance <1 nS.

Meanwhile, the protein suspension containing *T. californica* nAChRs, which was thawed at 4–8 °C overnight, was diluted by means of buffer C to 1 mg/ml. 100 μl of the diluted protein suspension were treated by sonication (1 burst, 50 W, 30 kHz, 1 mm diameter of the sonotrode, intensity 30%, cycle 0.5) using a tip sonicator (UP 50H, H, Hielscher, Teltow, Germany) followed by filtration through an 0.8 μm cellulose acetate filter. 10 μl were pipetted onto the SSM, avoiding any contact with the SSM-surface.

The sensor was sealed by a label (ToughSpot®, see above) and centrifuged at 2150×*g* and 20 °C for 2 h and then incubated at 4–8 °C overnight.

2.7. SSM-based electrophysiology

A typical protocol was described in detail by (Bazzone et al., 2013). In the present study, the protocol was adapted to specific needs with regard to compounds interacting with the nicotinic acetylcholine receptor in different functional states.

Before starting the measurements, all buffers were adjusted to room temperature in order to avoid artefacts. After removing the label, the sensors were placed in the SURFE^{2}R 500 workstation (Nanion, Munich, Germany) and rinsed by buffer C (100 mM NMG-H_2SO_4, 30 mM Tris, 3 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 by HCl). Capacitance and conductance were measured to check the quality of the protein vesicle-SSM complex. The adsorption of the protein vesicles onto the SSM was ideal when capacitance and conductance were similar to those of the sensors containing SSM only (∼3 nF and <1 nS, respectively). Each measurement series started with the incubation of the sensors with the blocking buffer C′ (buffer C that contained additionally 100 μM ambroxol hydrochloride) for 10 s (flow rate 100 μl/s) to block voltage-depended sodium channels (Na^+_V). Furthermore, each measurement series included positive and negative controls to detect signal rundowns due to protein degradation or loss of adsorption.

2.7.1. Positive and negative control

For the positive control, the sensor was rinsed by the non-activating buffer B (100 mM NaCl, 30 mM Tris, 3 mM EDTA, 3 mM EGTA, adjusted to pH 7.4 by 1 M HCl) for 2 s (flow rate 200 μl/s), followed by a rapid exchange with the activating buffer A (buffer B, which additionally contained 100 μM carbamoylcholine dichloride). After rinsing for 1 s (flow rate 200 μl/s), a rapid exchange back to the non-activating buffer and rinsing for 2 s (flow rate 200 μl/s) was performed. Accordingly, the measurement series "positive control" was finished and the series "negative control" was recorded. The negative control was performed analogously to the positive control except that the non-activating buffer B was used instead of the activating buffer A (Table 1).

2.7.2. Assay type "agonism"

For the determination of the dose-response curve, the concentrations of carbamoylcholine were modified (100 nM, 1 μM, 10 μM, 100 μM, 1 mM and 10 mM). The tested bispyridinium compounds MB327, MB583, MB781, MB779 and nAChR agonists nicotine and epibatidine were treated similar, regardless of their affinities.

2.7.3. Assay type "prevention"

To examine whether a desensitisation of the nAChR was prevented, the assay type "agonism" was modified. Additional to the increased carbamoylcholine concentration (see above), a constant concentration of the test compound (1 mM) was added.

2.7.4. Assay type "competition"

In order to determine whether the carbamoylcholine-induced signal was inhibited or enhanced, the carbamoylcholine concentration was kept constant (100 μM) and the concentration of the test compound was varied. As well as the assay types "agonism", "prevention" and positive and negative control (Table 1), the sensor was rinsed by the non-activating buffer B which forced the closed receptor-state.

2.7.5. Assay type "desensitisation"

To force the desensitised receptor state, the sensor was rinsed by the desensitisation buffer B' (non-activating buffer B, which contains additionally carbamoylcholine in excess (10 mM)). The sequence of the buffers was analogously to the negative control (Table 1).

2.7.6. Assay type "recovery"

In order to examine whether the substance was able to cancel the desensitisation and to recover the cholinergic signal, the rapid exchange was performed with the desensitisation buffer B' (10 mM carbamoylcholine, 100 mM NaCl, 30 mM Tris, 3 mM EDTA, 3 mM EGTA, adjusted to pH 7.4 by 1 M HCl) and the test compound was added in different concentrations (100 nM to 10 mM). A simplified overview of the setup is shown in Table 1.

The assay type described here allowed the induction of the receptor state "close" (assay types "agonism", "competition", "prevention") and the receptor state "desensitised" (assay type "recovery").

The first five measurements were carried out straight one after another and discarded. The initial non-evaluated measurements were needed to adjust the pH inside and outside of the protein vesicles. The transient capacitive currents were recorded in the time range 2–3 s and the signals were determined as peak height (nA). The specific cholinergic non-desensitised signal was defined as the difference between positive control (100%) and negative control (0%).

3. Results and discussion

3.1. Plasma membrane preparations

The plasma membrane preparations used in the present study were obtained after purification by means of sucrose density centrifugation (protein content 5.4 ± 1.3 mg/ml; $n = 6$, different batches). Previous experiments demonstrated that the bicinchoninic acid method was most advantageous, yielding recovery rates of ∼95%. However, reproducible results were obtained when the protein content was estimated before dilution with the buffer C that contained NMG salts. Estimation of diluted membrane

Table 1
Setups for the analysis of multiple activation states using plasma membrane fractions of *Torpedo californica*.

Assay type	Step 1 (t = 0–1 s)	Step 2 (t = 1–2 s)	Step 3 (t = 2–4 s)
Negative control	100 mM Na^+	100 mM Na^+	100 mM Na^+
Positive control	100 mM Na^+	100 mM Na^+ + 100 μM CCh	100 mM Na^+
Agonism	100 mM Na^+	100 mM Na^+ + 100 nM – 10 mM Agon	100 mM Na^+
Competition	100 mM Na^+	100 mM Na^+ + 100 μM CCh + 100 nM – 10 mM Comp	100 mM Na^+
Prevention	100 mM Na^+	100 mM Na^+ + 100 nM – 10 mM CCh + 1 mM Mod	100 mM Na^+
Desensitisation	100 mM Na^+ + 10 mM CCh	100 mM Na^+ + 10 mM CCh	100 mM Na^+ + 10 mM CCh
Recovery	100 mM Na^+ + 10 mM CCh	100 mM Na^+ + 10 mM CCh + 100 nM – 10 mM Mod	100 mM Na^+ + 10 mM CCh

CCh = Carbamoylcholine, Agon = Agonist, Comp = Competitor, Mod = Modulator, 100 mM Na^+ = non-activating buffer (100 mM NaCl, 30 mM Tris, 3 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 by 1 M HCl).

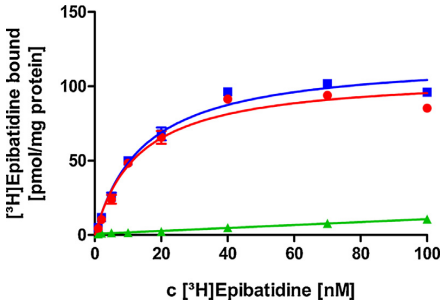

Fig. 2. Representative saturation isotherm of [^{3}H]epibatidine binding towards *Torpedo californica* membrane fractions. Data are shown as means ± SD (n = 3). Blue line, squares: total binding; green line, triangles: non-specific binding; red line; circles: specific binding. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

preparation resulted in decreased recovery rates (~50%). Obviously, NMG salts interfered with the components of the protein determination kit.

Furthermore, the plasma membrane preparation exhibited 94.5 ± 15.8 pmol/mg protein (n = 6; different batches) average maximum density of binding sites (B_{max}) referred to the agonist [^{3}H]epibatidine (Fig. 2). It should be noted that only the total number of binding sites without differentiation between high and low affinity binding sites were calculated. Obviously, [^{3}H] epibatidine is an agonist similar to carbamoylcholine, which is used in the present study, but it cannot be excluded that epibatidine and carbamoylcholine address different binding sites (Stock et al., 2014; Blum et al., 2010). The filtration assay performed for this study requires radio-labelled ligands, which bind in a high affinity (nanomolar equilibrium dissociation constants, K_D) due to the required high stability of the receptor–ligand complex during the filtration step. Even if radio-labelled carbamoylcholine had been available, the requirement would have been not fulfilled, because of its low affinity to nAChRs (K_D ~ 3 μM in mouse muscle adult type; (Akk and Auerbach, 1999)). Lack of high affinity carbamoylcholine derivatives made it necessary to choose [^{3}H] epibatidine (K_D ~ 15.4 ± 6.1 nM; n = 6). The quantification of [^{3}H] epibatidine binding sites directly on the sensor chip was not possible, because the exact amount of protein could not be determined after the supernatant of protein vesicles within the sensor cavity was washed-off. Besides, the ratio of inside-out and rightside-out orientation was random. Consequently, protein content and density of [^{3}H]epibatidine binding sites were theoretically estimated for each sensor. Nevertheless, standardised preparation protocols ensured that [^{3}H]epibatidine binding sites were an important tool to estimate the plasma membrane quality.

In combination with the protein content, the B_{max} value was important to adjust the receptor density on each sensor. At time of adsorption, each sensor exhibited ~10 μg protein, corresponding to ~80 fmol [^{3}H]epibatidine binding sites.

3.2. Sensor preparation

The rapid exchange of buffer was associated with high fluid mechanical effects onto the SSM and the adsorbed protein vesicle layer. Furthermore, the thickness of membranes seems to alter the energy barrier between resting, open and desensitised conformations (daCosta et al., 2013). The aim was the absorption of a monolayer of homogenous protein vesicles, whose size distribution was as constant as possible. In addition, particles with negatively charged surface may support the electrostatic attraction to the permanent positive charged surface of the SSM due to the emulsifier diphytanoyl-phosphatidylcholine. Therefore, the vesicles had to be characterised. Results are shown in Table 2.

It is known that vesicles with sizes 0.2–1 μm form stable monolayers (Papahadjopoulos and Vail, 1978). The size distribution expressed as polydispersity index (PI) should be as low as possible. Therefore, 0.45 and 0.8 μm cellulose membrane filters were tested for the preparation of standardised protein vesicles. The filtration through a 0.45 μm filter generated slightly smaller particle sizes (~270 nm). The treatment by 0.8 μm cellulose acetate produced an average particle size of ~290 nm. The PI of vesicles generated by filtration through 0.45 μm filters was higher than those of the vesicles produced by 0.8 μm filters (Table 2). With regard to particle size und particle size distribution, the filtration through 0.8 μm cellulose acetate filters was preferable.

However, crucial for the successful adsorption was a negative zeta potential. As mentioned above, the electrostatic attraction between the negatively charged vesicle surface and the positively charged SSM surface was possibly enhanced. The reconstitution of the plasma membrane obtained after sucrose density centrifugation in buffer C (30 mM Tris, 100 mM NMG-HCl, 3 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 by HCl) without filtration exhibited a zeta potential of zero and led to aggregation. Hence, the determination of Z_{avg} and PI could not be determined in this case. Negative zeta potentials were favourable for the adsorption of the protein vesicles onto the SSM. Reconstitution in storage buffer (10 mM sodium phosphate, 300 mM sucrose, 120 mM NaCl, 5 mM KCl, 0.5 mM EDTA) resulted in zeta potentials of significantly higher negativities than reconstitution in a Tris-/NMG-containing buffer (30 mM Tris, 100 mM NMG-HCl, 3 mM EDTA, 1 mM EGTA, pH 7.4). Thus, plasma membrane preparations obtained after sucrose density centrifugation were consistently reconstituted in storage buffer. Several purging cycles by sodium- and potassium-free buffers ensured decreasing of sodium and potassium ions within the vesicles. Exchange of the NMG-counterion (SO_4^{2-} instead Cl^-) resulted in less negative zeta potentials (−1.6 ± 2.6 mV versus

Table 2
Selected plasma membrane preparations and their vesicle properties. ZP, Z_{avg} and PI data are means ± SD (n = 10). Further details see text.

Buffer reconstitution	Buffer adsorption	Filtration d (μm)	ZP (mV)	Z_{avg} (nm)	PI
2	2	–	0.0 ± 0.2	Aggregation	–
2	2	0.8	−0.8 ± 1.5	298.9 ± 10.3	0.340 ± 0.026
1	2	0.8	−4.3 ± 1.1	290.6 ± 9.2	0.311 ± 0.037
1	2	0.45	−10.3 ± 1.9	268.8 ± 6.4	0.352 ± 0.054
1	2	0.8	−1.9 ± 0.6	343.4 ± 36.2	0.391 ± 0.046
1	3	0.8	−1.6 ± 0.6	278.1 ± 5.4	0.315 ± 0.034

d = average pore size of the cellulose acetate filter, ZP = zeta potential, Z_{avg} = average particle size, PI = polydispersity index.
Buffer 1 = 10 mM sodium phosphate, 300 mM sucrose, 120 mM NaCl, 5 mM KCl, 0.5 mM EDTA; pH 7.4.
Buffer 2 = 30 mM Tris, 100 mM NMG-HCl, 3 mM EDTA, 1 mM EGTA; pH 7.4 (1 M HCl).
Buffer 3 = 30 mM Tris, 100 mM NMG-H_2SO_4, 3 mM EDTA, 1 mM EGTA; pH 7.4 (1 M HCl).

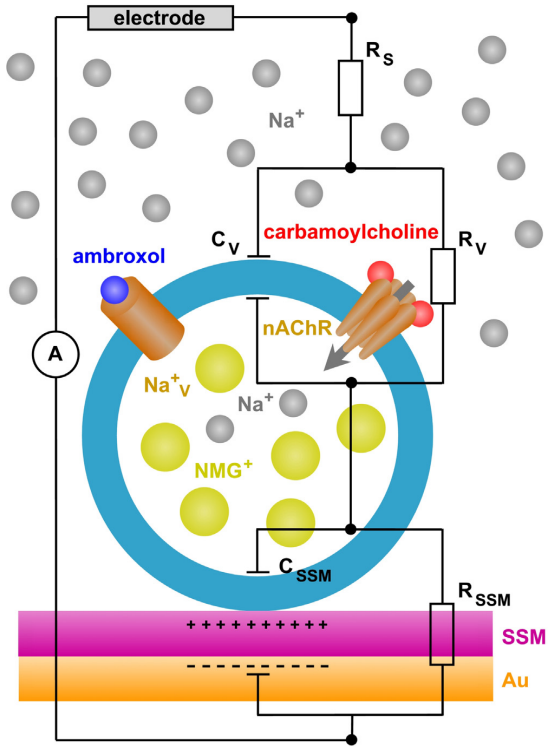

Fig. 3. The simplified circuit diagram illustrates the principle of capacitive coupling. The protein vesicle containing integral proteins (ion channels, transporters etc.) is adsorbed onto the solid supported membrane (SSM), which is attached on a gold surface (Au). Activation of nAChRs by the agonist carbamoylcholine generated a sodium influx in the protein vesicle. Voltage-dependent sodium channels (Na^+_V) were blocked by ambroxol.

−1.9 ± 0.6 mV), but the response in functional measurements was clearly higher (see next section). Plasma membrane preparations that have been filtered through 0.45 μm filters (average pore size), generally exhibited more negative zeta potentials (Table 2) than those treated by 0.8 μm filters.

However, the treatment by 0.45 μm filters was also associated with a high protein loss (up to 95%). Consequently, for the adsorption only 5% vesicle suspension remained, i.e. the sensors were loaded with 5% of the original amount of protein. The filtration using 0.8 μm filter membranes was more advantageous. Although protein loss with approximately 75% was still very high, the amount for adsorption seemed to be sufficient, expressing adequate resolution (~1 nA peak height).

In summary, reconstitution of the protein pellets obtained from sucrose density centrifugation in storage buffer and treatment by 0.8 μm cellulose-acetate filters was most advantageous for stable sensors.

3.3. SSM-based electrophysiology

The electrophysiological method used in this study was based on a capacitive-coupled membrane system formed by the SSM and a nAChR containing membrane. The charge translocation at the nAChR containing membrane was detected by capacitive coupling via the SSM. The protein vesicles contained barely sodium, but membrane-impermeable NMG^+ for charge compensation. Activation of the nAChRs was carried out by fast solution exchange (Fig. 3), the following Na^+-influx was triggered by the electrochemically gradient.

Replacement of NMG-HCl by NMG-H_2SO_4 resulted in better responses. Kosmotropic ions such as SO_4^{2-} have higher charge densities and therefore stabilise intramolecular interactions of proteins (Moelbert et al., 2004; Yang, 2009). However, complete elimination of chloride (e.g. use of Tris-H_2SO_4 and Na_2SO_4) was not feasible.

Besides nAChR, the plasma membrane preparations contained further integral proteins, e.g. voltage dependent sodium channels (Na^+_V). Previous experiments demonstrated that rinsing with the non-activating buffer B, containing among others 100 mM NaCl, indicated a premature collapse of the sodium gradient caused by Na^+_V. Consequently, at the time of carbamoylcholine addition nAChRs were activated but sodium influx stopped because the absent sodium gradient generated no driving force into the vesicle. An obvious disadvantage of SSM-based electrophysiology is that no voltage control over the Na^+_V is possible. Hence, Na^+_V had to be blocked by ligands in order to avoid a premature Na^+ gradient collapse. Therefore, the sensor was incubated for 30 min with the nonspecific Na^+_V blocker ambroxol (Weiser and Wilson, 2002). 1 μM of the more affine, but less soluble tetrodotoxin (Kao, 1986; Ulbricht et al., 1986) led to a similar result. In the present study, 100 μM ambroxol were sufficient to block the voltage-depended sodium channels.

3.4. nAChR activation

Rapid exchange with the activating, carbamoylcholine-containing buffer A indicated the open state of the nAChR, which enabled the transduction of sodium, represented as positive current (*y*-axis) (Fig. 4a). It has to be noted that the sodium influx cannot be measured directly. The capacitive current is the result of the sodium influx. Absence of the agonist carbamoylcholine at the time of activation had no effect (Fig. 4b).

For nAChR activation the agonist carbamoylcholine was chosen because the endogenic acetylcholine undergoes a non-enzymatic hydrolysis to acetate and choline during the measurement. In addition, enzymatic hydrolysis was more than likely because plasma membrane preparations used in this assay exhibited ~400 mU AChE activity/mg protein (Niessen et al., 2013). Possible interactions of AChE blockers with nAChRs, e.g. binding to still unknown binding sites cannot be excluded. Consequently, an AChE blocker was not used, as the tested substances including bispyridinium compounds were not affected by the AChE. Instead of acetylcholine, the hydrolysis-stable carbamoylcholine was chosen.

The signal of 100 μM carbamoylcholine was inhibited by the antagonist pancuronium, depending on its concentration. The bispyridinium compound MB779, bearing a C9 alkyl spacer and unsubstituted pyridinium rings (Fig. 1), showed a similar effect, although inhibition potency was lower (Fig. 4c). Both substances inhibited [^{3}H]epibatidine binding, pancuronium with higher affinity than MB779, whose affinity was micromolar (pancuronium pK_i 7.4 ± 0.1 and MB779 5.7 ± 0.0 respectively; n = 6) (Niessen et al., 2013). Interestingly, the addition of MB327 enhanced the cholinergic signal at low concentrations (Fig. 4d). Concentrations > 10 μM MB327 decreased the signal amplification. The addition of 10 mM MB327 exhibited no signal enhancement in comparison to the positive control (100 mM carbamoylcholine in absence of MB327). Recent *in vitro* experiments measuring muscle force by indirect electrical field stimulation (Seeger et al., 2012) showed a similar phenomenon. In this case, MB327 concentrations > 300 μM decreased the recovery of muscle force in soman-poisoned rat diaphragm. Lower MB327 concentrations enhanced the transient

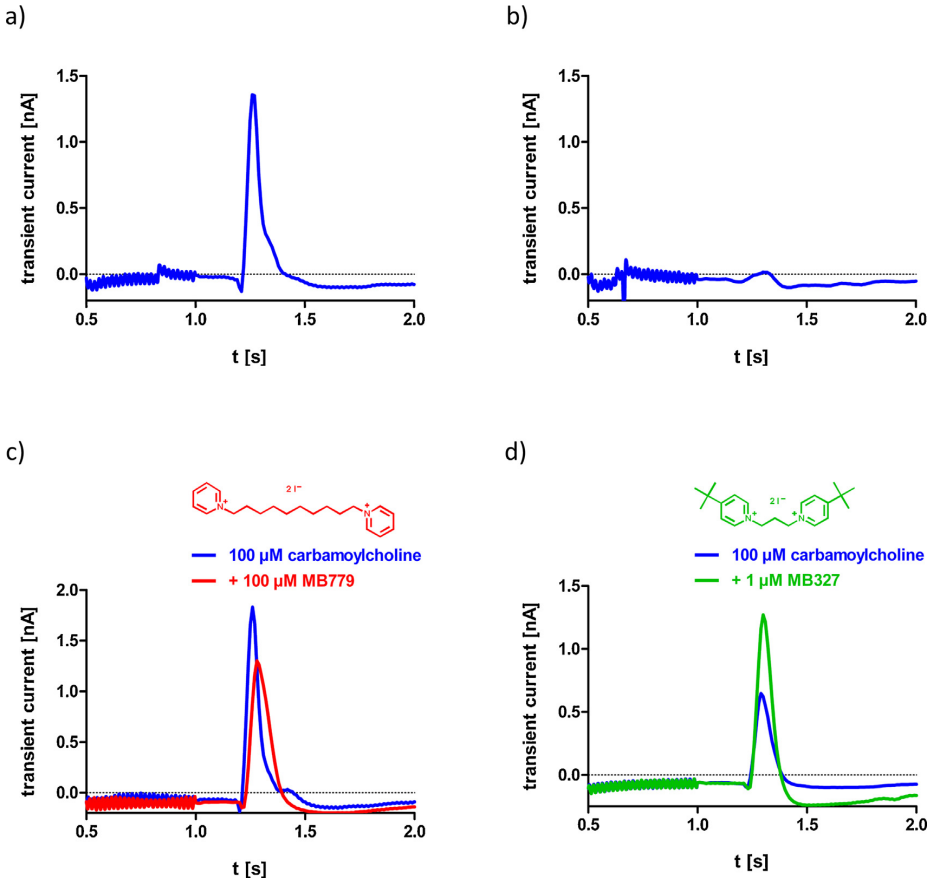

Fig. 4. Typical via capacitive coupling generated currents representing nAChR conductance activity. 100 µM of the agonist carbamoylcholine generated a cholinergic signal without loss of response, which was defined as positive control (a). Buffer without agonist showed no cholinergic effect (b). MB779 inhibited (c) and MB327 enhanced (d) the signal of 100 µM carbamoylcholine.

capacitive signal transduced by nicotinic acetylcholine receptors, whereas higher MB327 concentrations antagonised the cholinergic signal.

3.5. nAChR desensitisation

At low carbamoylcholine concentrations up to 100 µM the signal increased, indicating a clear dose-response relationship. However,

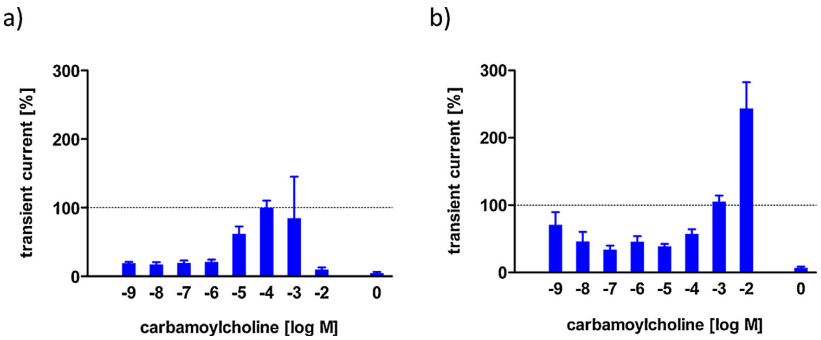

Fig. 5. Carbomoylcholine-induced desensitisation (a) and the prevention by 1 µM MB327 (b). Data are shown as means ± SD ($n = 4$) The positive control (defined as 100% transient current) is not shown.

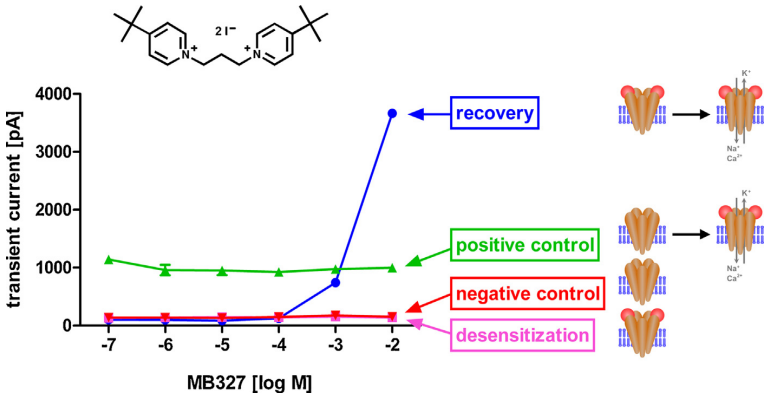

Fig. 6. Different activation states, performed with the same sensor. The concentrations refer to MB327. Further details see text. Data are shown as means ± SD ($n = 4$).

higher concentrations led to an abrupt extinction of the signal. The capacitive current of 10 mM carbamoylcholine was similar to the negative control (Figs. 4b and 5a). A premature collapse of the sodium gradient could be excluded, because subsequent measurements using 100 μM carbamoylcholine resulted in the same results as in previous cycles. Therefore, a carbamoylcholine-induced desensitisation of the nAChR had to be stated.

Interestingly, neither nicotine nor epibatidine caused a desensitisation under these experimental conditions due to possible different pharmacological profiles. As mentioned above, carbamoylcholine, nicotine and epibatidine may bind to different binding sites of muscle-type nAChRs (Kawai et al., 2008; Blum et al., 2013; Cashin et al., 2005). In comparison to human α7 nAChRs, carbamoylcholine demonstrated no regular inhibition of [^{3}H]epibatidine binding, albeit, the extent of the "abnormal" inhibition seemed to depend on divalent cations in the incubation buffer (Niessen et al., 2013). In addition, the activation mechanism of agonists at nAChRs seems to be differently. Experiments with α4β2 nAChR demonstrated that low concentrations of nicotine indicate a steady-state current (Papke et al., 2011), a phenomenon referred to as "smoldering" (Campling et al., 2013). Apparently, a lower barrier between the desensitisation state and the long-lived open state would allow the steady state current observed experimentally (Papke, 2014). Furthermore, there exist differences in the affinity towards nAChR-binding sites. The endogenous neurotransmitter acetylcholine shows 30-fold higher affinity towards the αδ binding site (Zhang et al., 1995), while carbamoylcholine shows lower affinity and lower site selectivity. The affinity of epibatidine towards the binding sites is opposite: 75-fold more preference for the αγ binding site (Prince and Sine, 1998a,b, 1999). Additionally, different opening times were observed. To elicit long opening times, binding of epibatidine solely to αγ binding site is sufficient (Stock et al., 2014). Probably, the differences in selectivity and affinity of the agonists carbamoylcholine, nicotine and epibatidine may explain that only carbamoylcholine induced the nAChR-desensitisation in this study.

When 1 μM MB327 was added to the activating buffer A and increasing concentrations of carbamoylcholine (1 nM–10 mM) were recorded, no carbamoylcholine-induced desensitisation was observed (Fig. 5b). In the assay type "prevention", the nAChRs were treated by the non-activating buffer in step 1 (Table 1). Hence, the absence of an agonist forced the closed state of the receptors. At the time of the rapid exchange with the agonist-containing buffer A (step 2), the pipetting of carbamoylcholine and MB327 into the cavity of the sensor chip was done simultaneously. At that moment, carbamoylcholine and MB37 competed for the binding sites of the formerly closed receptor. Apparently, the carbamoylcholine-induced desensitisation of the nAChRs was prevented by MB327.

It should be noted that MB327 induced no response in absence of the agonist carbamoylcholine (Fig. 5b). This finding is consistent with the pharmacological mechanism of a positive allosteric

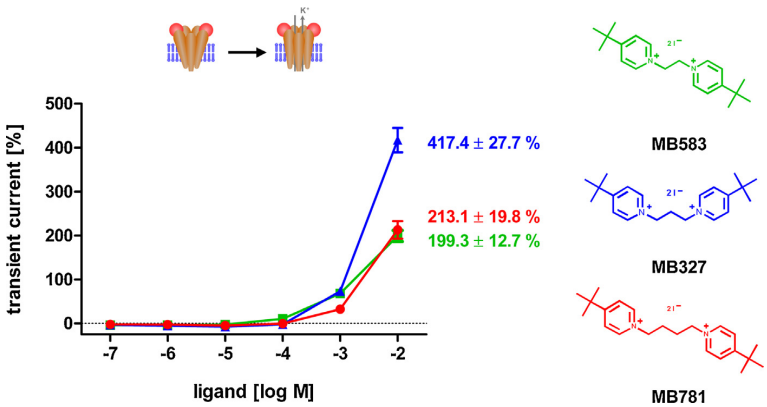

Fig. 7. Selected recovery profiles of three bispyridinium compounds bearing different long alkyl linkers. Further details see text. Data are shown as means ± SD ($n = 4$).

modulator (PAM). PAMs alone are not able to activate nAChRs, but they increase the activation efficacy/potency of nicotinic agonists (Williams et al., 2011).

3.6. Selective induction of conformations shifts

When searching for suitable antidotes for therapy of nerve agent poisonings, the desensitised receptor state is topic of interest, more precisely, the recovery of the desensitised nAChRs. Per concentration of the test compound the cycle "positive control–negative control–desensitisation control–recovery" was performed using the identical sensor. In the assay types "desensitisation" and "recovery", an excess of carbamoylcholine (10 mM) was used in all steps to assure desensitisation (Table 1).

In the assay type "desensitisation" the rapid exchange to the buffer B′ generated fluid mechanical peaks, which were similar to those of the negative control. The observed desensitisation was reversible because the following positive controls exhibited regular responses. During the complete assay, a slight increasing drift of the positive control was observed (Fig. 6).

Addition of MB327 generated a recovery of the cholinergic signal, when the concentration was 100 μM and higher. In contrast to the assay type "prevention", nAChRs were already treated by the desensitisation buffer B′ forcing the desensitised receptor state at the time of addition of the test compound MB327.

However, when positive and negative control was recorded, the closed but activatable receptor state, i.e. resting state was forced. Regarding the complete cycle (see above), alternate conformation shifts to the desensitised and resting state was manipulated, corresponding to the description that nAChR remain in the desensitised state with greatest probability until levels of agonist occupancy are reduced, following the diffusion or metabolism of the transmitter (Papke, 2014).

Correction by subtraction of the baseline (negative control and desensitisation, respectively) and normalisation to the positive control allowed the comparison of results generated by different sensors. Compared to structure-analogous bispyridinium compounds, bearing a shorter (C2; MB583) and a longer alkyl linker (C4; MB781), MB327 demonstrated more potency to recover the desensitised nAChR (Fig. 7). Further experiments performed with other structure-analogous bispyridinium compounds are in progress.

The observation of recovery is consistent with the pharmacological effects of type II PAM (Arias, 2010). In contrast to type I PAM, type II modulators decrease the population of desensitised receptors, probably by lowering the energy barrier between desensitised and open state (Williams et al., 2011).

4. Conclusions

The present results demonstrated that functional analysis of multiple activation states of nAChRs were successful by means of selective induction of conformation shifts.

In connection with affinity investigations, the functional measurements described here confirmed that bispyridinium compounds interact with nAChRs, although in a different manner. Small-sized bispyridinium compounds (C2–C4) were able to re-establish the activity of the desensitised nAChR. The effect of MB327, bearing a C3 alkyl linker, was most potent. Extended alkyl linkers (in this case C10) inhibited the cholinergic signal similar to pancuronium, but with less potency. It was shown that SSM-based electrophysiology is very useful in cases where conventional electrophysiology cannot be applied. However, the lack of voltage control especially over voltage gated cation channels (i.e. Na^+_V) was disadvantageous. Though, blocking of Na^+_V by ambroxol to avoid a premature sodium gradient collapse was a suitable corrective. Nevertheless, the crucial steps of the sensor preparation, particularly the preparation of the protein vesicles and adsorption of SSM and protein monolayer have to be implemented thoroughly. SSM-based electrophysiology is a very robust technique suitable for standardisation. Also due to the potential for automation SSM-based electrophysiology will find its place in drug development and drug discovery for new therapeutic approaches in the treatment of OPC-poisonings.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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In vitro pharmacological characterization of the bispyridinium non-oxime compound MB327 and its 2- and 3-regioisomers

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ABSTRACT

The primary toxic mechanism of organophosphorus compounds, *i.e.* nerve agents or pesticides, is based on the irreversible inhibition of acetylcholinesterase. In consequence of the impaired hydrolysis, the neurotransmitter acetylcholine accumulates in cholinergic synapses and disturbs functional activity of nicotinic and muscarinic acetylcholine receptors by overstimulation and subsequent desensitization. The resulting cholinergic syndrome will become acute life-threatening, if not treated adequately. The current standard treatment, consisting of administration of a competitive mAChR antagonist (*e.g.* atropine) and an oxime (*e.g.* obidoxime, pralidoxime), is not sufficient in the case of soman or tabun intoxications. Consequently, alternative therapeutic options are necessary. An innovative approach comprises the use of compounds selectively targeting nAChRs, especially positive allosteric modulators, which increase the population of the conducting receptor state. MB327 (1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium) di(iodide)) is able to restore soman-blocked muscle-force in preparations of various species including human and was recently identified as “resensitizer”. In contrast to the well-studied MB327, the pharmacological efficacy of the 2- and 3-*tert*-butylpyridinium propane regioisomers is unknown. As a first step, MB327 and its 3-regioisomer (PTM0001) and 2-regioisomer (PTM0002) were pharmacologically characterized using [^{3}H]epibatidine binding assays, functional studies by solid supported membranes based electrophysiology, and *in vitro* muscle-force investigations of soman-poisoned rat hemidiaphragm preparations by indirect field stimulation technique. The results obtained from targets of different complexity (receptor, muscle tissue) showed that the pharmacological profiles of the 2- and 3-regioisomers were relatively similar to those of MB327. Furthermore, high concentrations showed inhibitory effects, which might critically influence the application as an antidote. Thus, more effective drugs have to be developed. Nevertheless, the combination of the methods presented is an effective tool for clarifying structure-activity relationships.

1. Introduction

The verified homicidal use of the chemical warfare agent sarin in Syria 2017 (OPCW, 2017) illustrates that chemical warfare agents are still a serious threat for civilians and military personnel. The easy accessibility to highly toxic organophosphorus compounds (OPCs), *i.e.* nerve agents or pesticides, underlines the necessity for effective medical treatment.

The main acute toxic mechanism of OP agents is based on the inactivation of serine hydrolases such as acetylcholinesterase (AChE, EC 3.1.1.7) by phosphorylating the serine hydroxyl group located in the active site of the enzyme (MacPhee-Quigley et al., 1985; Taylor et al., 1995). By catalysing the hydrolysis, AChE terminates the intrinsic effect of the neurotransmitter acetylcholine at synaptic membranes and neuromuscular junctions. Upon inhibition of AChE, acetylcholine released from nerve terminals accumulates uncontrolled at peripheral and central cholinergic synapses, thereby disturbing functional activity of nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). The resulting cholinergic syndrome is characterized by peripheral (*e.g.* bronchorrhea, bronchospasm, muscular

Abbreviations: CNS, central nervous system; mAChR, muscarinic acetylcholine receptor; MB327, 1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium) di(iodide); nAChR, nicotinic acetylcholine receptor; NMG, N-methylglucamine; OPC(s), organophosphorus compound(s); PAM(s), positive allosteric modulator(s); PNS, peripheral nervous system; SSM, solid supported membrane(s); SURFE^{2}R, surface electronic event reader; PTM0001, 1,1′-(propane-1,3-diyl)bis(3-tert-butylpyridinium) di(iodide); PTM0002, 1,1′-(propane-1,3-diyl)bis(2-tert-butylpyridinium) di(trifluoromethanesulfonate); PTM0045, 1,1′-(propane-1,3-diyl)bis(2-tert-butylpyridinium) di(iodide); Tris, Tris(hydroxymethyl)aminomethane

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paralysis, salivation, involuntary defecation and urination) and central effects (*e.g.* tremor, seizures, disturbance of respiratory drive) (Gunderson et al., 1992; Marrs, 1993). Lethality is finally caused by peripheral and central respiratory arrest (Grob, 1956; Rickett et al., 1986).

As symptoms may develop extremely fast, treatment must be started as soon as possible. Therefore, prompt administration of specific antidotes is crucial to increase survival (Newmark, 2004). The current standard drug therapy of OPC intoxication consists of the administration of the competitive mAChR antagonist atropine and oximes (*e.g.* obidoxime, pralidoxime) for reactivating inhibited AChE (Thiermann et al., 1999, 2007). Additionally, anticonvulsants are given to alleviating acetylcholine mediated seizures (Shih et al., 2007). However, no ingredients that directly mitigate symptoms related to nAChRs exist so far.

That definitive gap in the treatment of OPC poisoning may be a serious problem when AChE inhibited by certain OPCs can hardly be reactivated by commonly used oxime AChE reactivators in case of tabun (Ekström et al., 2006), or enzyme-organophosphate complexes undergo rapid dealkylation reaction (so-called “aging”) as *e.g.* in the case of soman (Worek et al., 2005a,b; Sirin et al., 2012), thereby preventing reactivation (Worek et al., 2004). Finally, peripheral respiratory arrest due to neuromuscular block is a major problem and calls for approaches to restore neuromuscular transmission at respiratory muscles (Thiermann et al., 2010). In these cases, drugs selectively targeting nAChRs, particularly with the aim to restore endogenous cholinergic tone to ensure survival despite of ongoing inhibition of AChE are of therapeutic interest.

The nicotinic acetylcholine receptor (nAChR) is a cation selective, pentameric neurotransmitter-gated ion channel (Unwin, 2013) and a member of the so-called “Cys-Loop family” (Lester et al., 2004; Sine and Engel, 2006; Corringer et al., 2012). nAChRs are widely distributed in the brain, in both sympathetic and parasympathetic ganglia, in skeletal muscles, in epithelial and immune cells (Kalamida et al., 2007), and are expressed in both pre- and postsynaptic locations of the synaptic cleft (Wu and Lukas, 2011). They play important functional roles in cholinergic neurotransmission in both the peripheral (PNS) and central nervous system (CNS) and are implicated in different CNS disorders, *e.g.* Alzheimer’s and Parkinson’s disease, schizophrenia, depression, tobacco addiction, and PNS disorders, *e.g.* congenital myasthenic syndrome, myasthenia gravis (Gotti and Clementi, 2004; Melzer et al., 2016). Consequently, drugs that modulate nAChR functions are becoming increasingly important (Hogg and Bertrand, 2004; Gündisch, 2005; D’Hoedt and Bertrand, 2009; Hurst et al., 2013), especially for the treatment of intoxications, *e.g.* by organophosphorus compounds (OPCs) (Mamczarz et al., 2011). Conventional nAChR antagonists are not appropriate, because of their low therapeutic index between sufficient antagonism and muscle paralysis (Sheridan et al., 2005). Rather, positive allosteric modulators (PAMs), whose effects would not be overwhelmed by increasing concentrations of agonist (*e.g.* acetylcholine), might be an alternative approach for the therapy of OPC poisoning.

Previous studies have demonstrated that bispyridinium compounds, which were lacking the oxime group, induced a significant improvement of neuromuscular transmission which was not related to conventional reactivation of the inhibited AChE enzyme (Tattersall, 1993).

Indirect electric field stimulation experiments with soman-poisoned human intercostal muscle and rat diaphragm preparations confirmed that the non-oxime bispyridinium compound MB327 (1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium) di(iodide)) was able to restore the neuromuscular function. This effect was attributed to a direct pharmacological action on the muscle (Seeger et al., 2012).

Meanwhile, a receptor-mediated effect of MB327 was confirmed by affinity and functional assays. Depending on the linker length, symmetric bispyridinium compounds interact directly with nAChRs by influencing the binding of the highly affine agonist [^{3}H]epibatidine (Niessen et al., 2011, 2013). MB327 may increase the affinity of [^{3}H] epibatidine to muscle-type nAChRs ($\alpha\beta\delta\gamma$, *Torpedo californica*) and enhance carbamoylcholine-evoked signals, which was shown in functional studies based on solid supported membranes (SSM)-based electrophysiology. Additionally, MB327 was able to recover the activity of carbamoylcholine-desensitized nAChRs in a typical type II PAM manner (Niessen et al., 2016).

However, efficacy of MB327 was found only in the millimolar range, making a practicable application as therapeutic approach difficult. Additionally, MB327 is not subtype-selective (Ring et al., 2015) and antagonizes mAChRs (Königer et al., 2013).

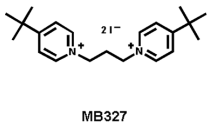
In contrast to the well-studied MB327, the pharmacological effects of the 2- and 3-*tert*-butylpyridinium regioisomers are unknown so far. As first step of a medicinal chemistry program, the 3-regioisomer (PTM0001) and 2-regioisomer (PTM0002) in comparison of the lead structure MB327 were pharmacologically characterized using [^{3}H]epibatidine affinity assays, functional studies by SSM-based electrophysiology and *in vitro* muscle-force investigations of soman-poisoned rat hemidiaphragm preparations by indirect field stimulation. The principles of these methods are described in detail (Niessen et al., 2011; Schulz et al., 2008; Seeger et al., 2007). Affinity and functionality assays were carried out with plasma membranes from the *Torpedo californica* electric organ, a rich source of the $\alpha\beta\gamma\delta$ nAChR subtype (Whittaker, 1989). Although the *Torpedo* nAChR is not 100% identical to the human muscle-type isoform, both show a high degree of homology (Millar, 2003; Navedo et al., 2004). Appropriate plasma membrane preparations containing human adult $\alpha1\beta1\delta\varepsilon$-nAChRs were not available.

The aim of this study was to determine potential differences between MB327 and its 2- and 3-regioisomers. Furthermore, the analysis of the individual results obtained from various complex targets (receptor, tissue) should allow an assessment whether the different results obtained are part of a common picture. Finally, the study should confirm that the methods in combination are useful for clarifying meaningful structure-activity relations.

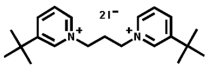
2. Material and methods

2.1. Materials

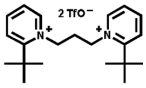
Male Wistar rats (300 g ± 50 g) were obtained from Charles River, Sulzfeld, Germany, *Torpedo californica* electroplaque tissue was purchased from Aquatic Research Consultants (San Pedro, CA, USA). Soman (> 93% by ^{1}H and ^{31}P NMR) was made available by the German Ministry of Defence. Epibatidine, [5,6-cycloheptyl-^{3}H] with a specific activity of approximately 2 TBq/mmol and disposables, such as filtermates and solid scintillators, were obtained from Perkin Elmer (Jügesheim, Germany). (±)-Epibatidine was purchased from Tocris (Bristol, UK). Pancuronium bromide, carbamoylcholine dichloride and other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). Polypropylene microtiter plates, tubes and tips were obtained from Eppendorf (Hamburg, Germany). All other chemicals in the purest available grade were purchased from E. Merck (Darmstadt, Germany). The tested bispyridinium compounds MB327, PTM0001, PTM0002 (chemical structures see Fig. 1) were synthesized by a newly developed synthetic strategy (Rappenglück et al., 2017) in ≥99% purity measured by ^{1}H qNMR spectroscopy and mass spectrometry. PTM0045 (iodide salt of PTM0002) was synthesised for investigation of possible effects due to the different counterions. Stock solutions of bispyridinium compounds, pancuronium bromide, (−)-nicotine hydrogen tartrate and carbamoylcholine dichloride were prepared in distilled water (1–10 mM), “desensitizing” buffer (see Section 2.3) or Tyrode solution (see Section 2.4), respectively, and (±)-epibatidine in ethanol (1 mM).



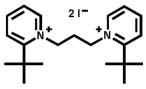
MB327



PTM0001



PTM0002



PTM0045

Fig. 1. The non-oxime bispyridinium compound MB327 and its 3-regioisomer PTM0001 and 2-regioisomers PTM0002, PTM0045.

Table 1
Composition of buffers used in the setup "recovery" of SSM-based electrophysiology.

	Composition
Buffer B	100 mM NaCl 30 mM Tris–HCl 3 mM EDTA 1 mM EGTA
Buffer A	100 mM NaCl 30 mM Tris–HCl 3 mM EDTA 1 mM EGTA 100 µM carbamoylcholine dichloride
Buffer D	100 mM NaCl 30 mM Tris–HCl 3 mM EDTA 1 mM EGTA 10 mM carbamoylcholine dichloride
Buffer R	100 mM NaCl 30 mM Tris–HCl 3 mM EDTA 1 mM EGTA 10 mM carbamoylcholine dichloride 10 nM–1 mM (10 mM) tested compound

2.2. Radioligand binding

Radioligand binding experiments using [^{3}H]epibatidine were carried out on a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany) and performed as previously described (Niessen et al., 2013). In short, membranes from the frozen electric organ of *Torpedo 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 buffer [10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl, 0.1 mM PMSF (freshly added), 1 pill EDTA-free protease inhibitor combination per 50 ml ("Complete® EDTA free", Roche, Grenzach-Wyhlen, Germany); pH 7.4] was stored at −150 °C until use. The purified membranes were used in radioligand binding experiments as well as in functional measurements performed with SSM-based electrophysiology.

All radioligand binding assays were performed in assay binding buffer (10 mM sodium phosphate buffer, 120 mM NaCl, 5 mM KCl, pH 7.4) using 96-deep well plates. Dilution of the *Torpedo* membranes, incubation, filtration and detection were performed according to a previously described method (Niessen et al., 2013).

In competition assays, the concentration of [^{3}H]epibatidine was 3 nM and of the tested bispyridinium compounds 100 nM–1 mM (six replicates per concentration). Non-specific binding was determined in the presence of 10 µM unlabelled (±)-epibatidine. For estimation of K_i values three independent experiments were performed.

K_i values were calculated with nonlinear regression (Prism 5.0, GraphPad Software, San Diego, CA, USA) obtaining sigmoidal dose–response curves (One site—Fit Ki).

The top and bottom of the sigmoidal curve were constrained to values obtained for total binding (without competitor, top) or non-specific binding (in the presence of 10 lM epibatidine, bottom).

2.3. SSM-based electrophysiology

Protein vesicles were prepared and characterized by radioligand saturation assays using the agonist [^{3}H]epibatidine, and by photon correlation spectroscopy (PCS) to ensure consistent quality of the protein vesicles adsorbed onto the sensor chips. SSM-based electrophysiology carried out on the SURFE^{2}R 500 workstation and partially on the high-throughput system SURFE^{2}R 96SE (Nanion, Munich, Germany) was performed as described previously (Niessen et al., 2016).

For investigation of PAM type II pharmacology, a special protocol was used. The setup was focused on the assay type "desensitization" to elucidate the potency of chemical compounds to recover cholinergic signals of nAChRs in their desensitisation state induced by an excess of the agonist carbamoylcholine.

Briefly, the sensors were rinsed by a washing buffer (100 mM NMG-H_2SO_4, 30 mM Tris, 3 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 with HCl) after equilibration of all components at ~20 °C. Before starting each measurement series, the sensors were incubated with a blocking buffer (washing buffer that contained additionally 100 µM ambroxol hydrochloride) for 10 s (flow rate 100 µl/s) to block voltage-dependent sodium channels (Na_V). The setup included positive and negative controls to detect signal rundowns due to protein degradation or loss of adsorption. For the positive control, the sensor was rinsed by the non-activating buffer "B" (composition see Table 1) for 2 s (flow rate 200 µl/s), followed by a rapid exchange with the activating, nAChR agonist containing buffer "A" (Table 1). After rinsing for 1 s (flow rate 200 µl/s), a rapid exchange back to the non-activating buffer "B" and rinsing for 2 s (flow rate 200 µl/s) followed. The negative control was performed analogously to the positive control except that the activating buffer "A" was replaced by the non-activating buffer "B".

Periodically, the quality of the SSM (with and without adsorbed protein vesicles) was checked by comparing capacitance and conductance with the optimal values (~30 nF and < 1 nS, respectively).

To force the desensitized receptor state, sensors were rinsed with the buffer "D" (Table 1), containing the nAChR agonist carbamoylcholine in excess ("desensitizing buffer"). Analogously to the negative control, the setup "desensitization" was performed with rapid buffer exchanges at 2 and 3 s after sequence start. The "recovering buffer" (buffer "R", Table 1) contained additionally the test compounds in different concentrations (10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM; partially 10 mM, if dissolvable). Analogously to the positive control, the sensor was rinsed with the "desensitizing buffer" for 1 s, rapidly replaced with the "recovering buffer" and washed out again by the "desensitizing buffer" (3 s). Table 2 shows the complete setup performed on each sensor.

The transient capacitive currents generated by inward cation flux into the vesicles were determined as peak height (nA). The specific cholinergic signal recorded in the time range 2–3 s (Table 2) was defined as the difference between positive control (100%) and negative control (0%).

Table 2
Buffer exchange performed in the setup "recovery" of SSM-based electrophysiology.

	0–2 s	2–3 s	3–5 s
Positive control	B	A	B
Negative control	B	B	B
Desensitization	D	D	D
Recovery	D	R	D
Negative control	B	B	B
Positive control	B	A	B

2.4. Rat diaphragm myography

All procedures using animals followed animal care regulations and were approved by the responsible ethics committee.

Preparation of rat diaphragm hemispheres and experimental protocol of myography was performed as described before with slight modifications (Seeger et al., 2007, 2012). In short, for all procedures (including wash-out steps, preparation of soman and bispyridinium compound solutions) aerated Tyrode solution (125 mM NaCl, 24 mM $NaHCO_3$, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 95% O_2, 5% CO_2; pH 7.4; 25 ± 0.5 °C) was used. After recording of control muscle force, the muscle preparations were incubated in the Tyrode solution, containing 3 µM soman. Following a 30 min wash-out period the test compounds MB327, PTM0001 or PTM0002 were added in ascending concentrations (1 µM, 10 µM, 30 µM, 100 µM, 300 µM, 1 mM). The incubation time was 30 min for each concentration. The electric field stimulation was performed with 5 µs pulse width and 2 A amplitudes. The titanic trains of 20 Hz, 50 Hz, 100 Hz were applied for 1 s and in 10 min intervals.

Muscle force was calculated as a time-force integral (area under the curve, AUC) and constrained to values obtained for maximal force generation (muscle force in presence of Tyrode solution without any additives; 100%).

2.5. Data and statistical analysis

All results were expressed in means ± SD (n = 3–22). One-way analysis of variance (ANOVA) and Bonferroni post-analysis was employed to reveal significant differences ($p < 0.05$). For all data analysis, Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used.

3. Results

3.1. Effects on muscle-type nAChRs

3.1.1. Affinity

[^{3}H]Epibatidine binding experiments in presence of MB327 and PTM0001 (both iodide salts) exerted two different, overlapping effects (Fig. 2). Firstly, an increasing affinity of the orthosteric agonist [^{3}H] epibatidine in dependence on the bispyridinium compound concentrations. Secondly, after reaching the maxima (~130% and ~125% specific binding induced by MB327 and PTM0001, respectively) an inhibition of [^{3}H]epibatidine binding at concentrations > 100 µM was observed. In the case of the triflate salt PTM0002, the phenomenon of affinity increase was less pronounced (~115%). The results of the iodide salt PTM0045 were similar to those of PTM0002, respectively (data no shown). All tested compounds increased the affinity and displaced [^{3}H]epibatidine in a "non-classical" manner. In contrast, the nonlabelled (±)epibatidine displaces the radiolabelled marker [^{3}H] epibatidine in a regular concentration dependency (Fig. 2).

3.1.2. Functional effects

In the SSM-based electrophysiology charge shifts of a serial coupled capacitor were recorded. The capacitor system on the sensor chip consisted of a lipid-emulsifier membrane system (SSM) and a layer of protein vesicles, whose membrane is containing integral nAChRs (Schulz et al., 2009; Niessen et al., 2016). Inside the protein vesicles only low sodium concentrations are present, but membrane-impermeable NMG^+ for charge compensation. In this assay, activation of the nAChRs induced a sodium influx triggered by the electrochemically gradient and a subsequent charging of the serial coupled capacitor. Finally, the charge compensation was recorded as a transient current. In summary, the interaction of ligands with the nAChR and the hereby evoked functional change of the receptor are detected indirectly *via* charge translocation.

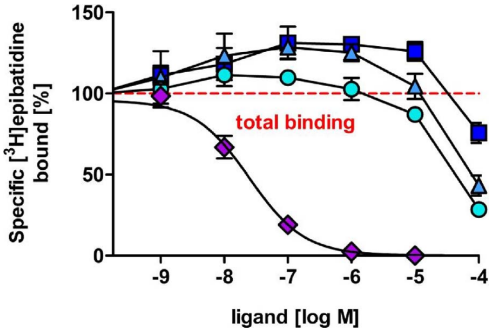


Fig. 2. Selected competition assays of MB327 (sqares), PTM0001 (triangles), PTM0002 (circles) and (±)epibatidine (rhombi). Total binding was determined without ligand and non-specific binding in the presence of 10 µM (±)epibatidine. Data are means ± SD (n = 6).

For testing type II PAM potency, the functional recovery ("resensitizing") of desensitized nAChRs induced by an excess (10 mM) of the agonist carbamoylcholine was investigated (Fig. 3).

The results of MB327 und PTM0001 were quite similar. The activity of the previously desensitized nAChRs was recovered by applying high concentrations of the bispyridinium compounds. All values are normalized to 100%, *i.e.* the response in presence of 100 µM carbamoylcholine. At this concentration no desensitization is observed, suggesting that the receptor is in its normal functional state. The concentration of 1 mM resulted in 73.0 ± 6.5% recovery for MB327 and 72.0 ± 7.7% recovery for PTM0001. 10 mM MB327 exerted 417.2 ± 9.3% recovery, whereas the preparation of 10 mM PTM0001 was not successful because of its limited solubility.

The 3-regioisomer PTM0002 demonstrated a different effect: 1 mM generated no marginal resensitizing of receptor activity (2.5 ± 0.6%), which slightly increased when 10 mM were applied (14.9 ± 6.1%).

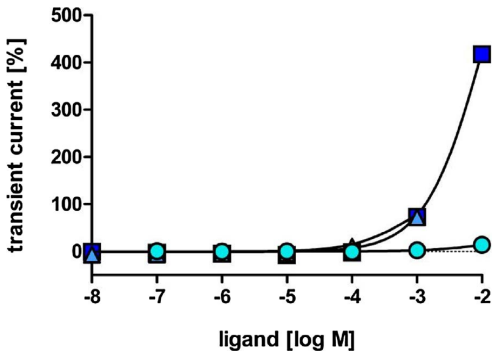


Fig. 3. Functional measurements by SSM-based electrophysiology. Selected recovery profiles of MB327 (sqares), PTM0001 (triangles), PTM0002 (circles). The preparation of 10 mM PTM0002 was not successful because of its limited solubility. Data are means ± SD (n = 4–6).

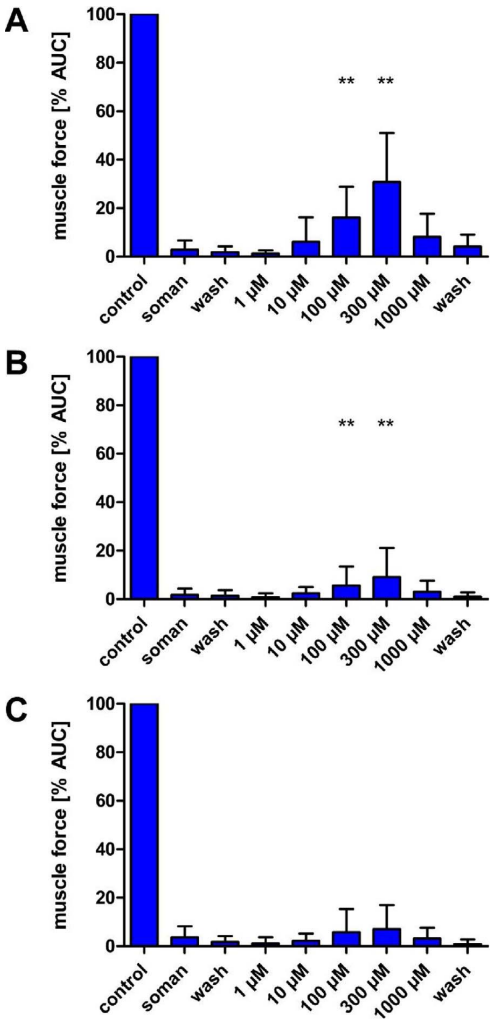


Fig. 4. Effect of MB327 on soman-impaired neuromuscular transmission. The recovery of muscle force (rat diaphragm hemispheres) is expressed as AUC constrained to normal activity (control, 100%). For indirect stimulation frequencies of A) 20 Hz, B) 50 Hz, C) 100 Hz were applied. Data are shown as mean ± SD (n = 27). Asteriks indicate significant differences between MB327 and first wash-out (* $p < 0.05$; ** $p < 0.01$).

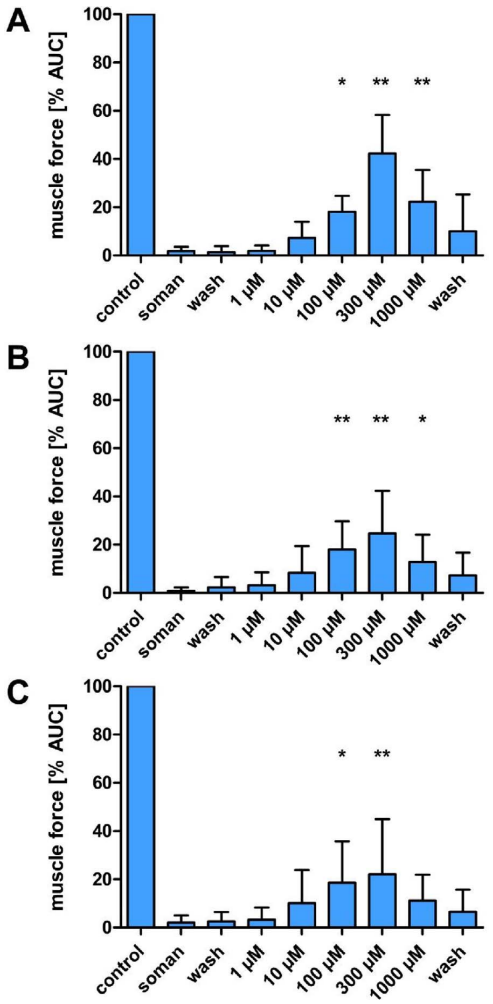


Fig. 5. Effect of PTM0001 in dependence of concentration. The recovery of muscle force (rat diaphragm hemispheres) is expressed as AUC constrained to normal activity (control, 100%). For indirect stimulation frequencies of A) 20 Hz, B) 50 Hz, C) 100 Hz were applied. Data are shown as mean ± SD (n = 6). Asteriks indicate significant differences between PTM0001 and first wash-out (* $p < 0.05$; ** $p < 0.01$).

PTM0002 differs from MB327 and PTM0001 in the counterion: triflate instead of iodide. Thus, it cannot be ruled out that the agonist induced charge translocation was masked by triflate counterions in this setup. However, parallel experiments with PTM0002 and the iodide salt PTM0045 showed no significant difference in the results. Additionally, repeated measurements confirmed the marked difference between the 3-regioisomer PTM0002 and the active substances MB327 and PTM0001.

In summary, although clear differences between PTM0002 and MB327/PTM0001 were observed, no *tert*-butylpyridinium propane regioisomer exerted efficacy in the micromolar or even nanomolar range.

3.2. Muscle force generation

Muscle contraction induced by indirect electrical field stimulation was almost completely blocked after application of 3 µM soman at tetanic stimulation frequencies of 20, 50 and 100 Hz. This effect could not be antagonized by washing out (Figs. 4–6) and indicated irreversible inhibition of AChE.

When MB327 was added, a concentration-dependent recovery of soman-impaired muscle force was recorded. After reaching maxima, increasing concentrations (> 300 µM) reduced the extent of recovery (almost baseline). The extent of recovery depended on the stimulation frequency and was most pronounced at 20 Hz (maximal 31 ± 4% normalized muscle force). Stimulation frequencies of 100 Hz generated less than 10% maximal recovery of muscle force (Fig. 4).

The effects of the 3-regioisomer PTM0001 and 2-regioisomer PTM0002 showed a similar pattern as MB327. PTM0001 and PTM0002 exerted concentration-dependent muscle force recovery, reaching a maximal effect, when 300 µM was applied. Applying 20 Hz, maximal amplitudes of 42 ± 6% and 43 ± 6% were recorded for PTM0001 and PTM0002, respectively (Figs. 5 and 6). Analogously to MB327, increasing stimulation frequencies resulted in a lower recovery of

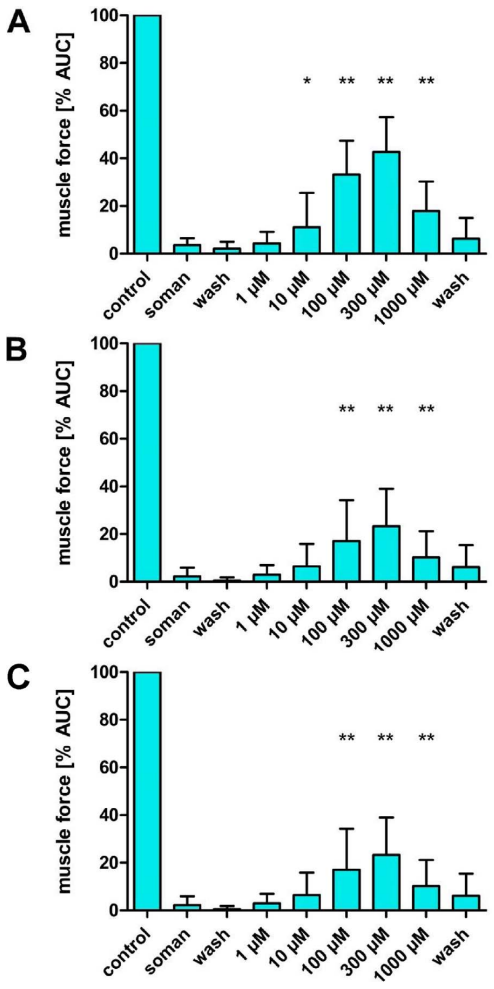


Fig. 6. Effect of PTM0002 in dependence of concentration. The recovery of muscle force (rat diaphragm hemispheres) is expressed as AUC constrained to normal activity (control, 100%). For indirect stimulation frequencies of A) 20 Hz, B) 50 Hz, C) 100 Hz were applied. Data are shown as mean ± SD (n = 20). Asteriks indicate significant differences between PTM0002 and first wash-out (* $p < 0.05$; ** $p < 0.01$).

muscle force generation in presence of PTM0001 and PTM0002. Remarkably, PTM0001 generated higher amplitudes (22 ± 9%) than PTM0002 (14 ± 3%) and MB327 (7 ± 2%) at 300 µM (maximal effect) after 100 Hz stimulation (Fig. 5).

In all experiments, the pharmacological effect of the bispyridinium compounds was eliminated by a subsequent wash-out step. The removal of the bispyridinium compounds by washing-out indicated that the recovering of muscle force was receptor-mediated and not related to an enzyme-catalysed hydrolytic process, *i.e.* reactivation of soman-inhibited AChE.

4. Discussion

4.1. nAChR-mediated effects

The bispyridinium non-oximes tested in this study influence the binding of the highly affine orthosteric agonist [^{3}H]epibatidine. A regular displacement of [^{3}H]epibatidine as expected for "classical" orthosteric ligands was not observed. Rather, MB327 and its 3- and 2-regioisomers PTM0001 and PTM0002, respectively, induced an increasing affinity of [^{3}H]epibatidine, which indicates an allosteric effect. Thus, the interaction with nAChRs can only be detected indirectly, because a binding site different from the orthosteric epibatidine binding site is addressed. For assessment of precise affinities of bispyridinium structure analogues the use of the well described MB327 appears rational. However, radiolabelled MB327 is not available and, furthermore, lacks high affinity.

In summary, the binding assay used in this study does not provide affinity constants for the tested bispyridinium compounds regarding their binding sites. The displacement of the orthosteric agonist [^{3}H]epibatidine in a "regular" manner was not observed for all three *tert*-butylpyridinium propane regioisomers. Rather, indications for allosteric interactions with the nAChRs were shown.

In poisoning by soman or tabun, a reactivation of inhibited AChE is therapeutically hardly achievable. Consequently, the disturbed neuromuscular transmission caused by receptor desensitization and therefore the recovery of desensitized nAChRs by positive allosteric modulators is a progressively interesting topic. A newly developed functional assay based on solid supported membranes (SSM) is a valuable tool to assess the potency of PAMs type II, a ligand class that probably lowers the energy barriers between desensitized and open receptor states (Williams et al., 2011).

Comparing structure-analogous bispyridinium compounds, possessing alkyl spacers of varying length, the *n*-propyl possessing bispyridinium compound MB327 demonstrated the highest potency to recover the activity of desensitized nAChR (Niessen et al., 2016). However, it was not known whether different substitution patterns of the pyridinium moieties affect the potency of recovery.

Addition of MB327 and PTM0001 generated a recovery of the cholinergic signal, when the concentration was 1 mM and higher. In contrast, only a slight recovery effect of PTM0002 was shown, even when high concentrations were applied (10 mM). As mentioned before, MB327 and PTM0001 are iodide salts and PTM0002 is a triflate salt, which led to the assumption that the different counterions could be contributed to the different response. This suggestion was not confirmed by direct comparison of the effects of PTM0045 and PTM0002.

For SSM-based electrophysiology, a plasma membrane preparation from the electric organ of the pacific ray, a rich source of nAChRs (Whittaker, 1989), was used. Desensitization of nAChRs was only achieved by applying high agonist concentrations (10 mM carbamoylcholine) (Niessen et al., 2016). For recovering nAChR activity in the functional screening, concentrations up to 1 mM (partially 10 mM) could be applied for MB327, PTM0001 and PTM0002 due to their limited solubility. In view of the high density of binding sites and high carbamoylcholine concentrations no saturation (or even inhibition) analogously to the effects on human α7 nAChRs (Scheffel et al., 2017) could be observed, thus preventing estimation of valid EC_{50}. The effects of the bispyridinium compounds are based on a classical ligand-receptor-interaction and resulting ligand binding stabilizes a specific receptor state ("resensitized"). In fact, ligand-receptor-interaction follows the law of mass action (Motulsky and Mahan, 1984), more precisely, the relations between density of binding sites, the amount of the agonist and the allosteric modulator and furthermore, the affinities of the ligands to their binding sites. Especially the low response of PTM0002 indicates that higher concentrations (*i.e.* > 10fold of carbamoylcholine) probably result in higher populations of "resensitized" open states.

In the end, this method allows the detection of highly effective substances—a property which was not shown for PTM0002, and for MB327 and PTM0001 only after applying high concentrations (≥ 1 mM).

4.2. Pharmacological effects on muscles

The setup of the assay ensured that the effects of the tested

bispyridinium compounds were examined after ageing of soman inhibited AChE (Talbot et al., 1988). The regioisomers PTM0001 and PTM0002, as well as MB327, were able to restore muscle force in a concentration-dependent manner without reactivating soman-inhibited AChE. Previous experiments including enzyme activity measurements in muscle biopsies confirmed that soman-inhibited AChE was not reactivated by MB327 (Seeger et al., 2012). Additionally, the observed positive effects could be washed out, indicating a direct interaction with nAChRs and not an enzyme reactivation.

MB327, PTM0001 and PTM0002 were able to improve neuromuscular transmission after soman poisoning in presence of accumulated acetylcholine, most likely resulting in desensitized nAChRs. On base of this assumption, the therapeutic effect of the test compounds may be attributed to the recovery of the activity (*i.e.* "resensitizing") of desensitized nAChRs, which are typical properties of type II PAMs.

Various studies postulated that recovery of 5–20% muscle force after OPC poisoning may be sufficient to enable survival (Thiermann et al., 2009; Seeger et al., 2012). A positive therapeutic effect of MB327 in nerve agent poisoning was confirmed in several *in vivo* experiments. For example, the LD_{50} of soman was increased in a dose-dependent manner under treatment of MB327 in combination with atropine and anticonvulsant in guinea-pigs. The survival of the animals was statistically significant ($p < 0.01$), when a dose of 34 mg kg^{-1} was administered (Price et al., 2016). *Ex vivo* studies with soman-poisoned rat diaphragm hemispheres and human intercostal muscle preparations showed a comparable effect of MB327 on restoring muscle force which may indicate the possibility to transfer animal data to humans (Seeger et al., 2012).

Interestingly, higher concentrations of the bispyridinium compounds (> 300 μM) resulted in a reduction of the therapeutic effect in the muscle force experiments. This effect was observed for all three regioisomers and may indicate an inhibition of nAChRs in the synaptic cleft. Obviously, the therapeutic effect is overlapped by a secondary inhibitory effect manifested at higher concentrations. The bispyridinium compounds probably interact with further binding sites stabilising a non-conducting receptor state, but quantity and locations of the addressed binding sites are unknown at present.

In summary, micromolar concentrations of the three tested *tert*-butylbispyridinium propane regioisomers are needed for positive therapeutic effects and higher concentrations mitigate these effects.

5. Conclusions

The present study analysed the pharmacological effects of MB327 and its 2- and 3-regioisomeres with three different methodologies, *i.e.* radioligand binding, SSM-based electrophysiology and rat diaphragm myography.

An inhibition of [^{3}H]epibatidine binding by the bispyridinium compounds indicating a direct interaction with orthosteric binding sites could not be observed at low micromolar concentrations. Rather, the increasing affinity of [^{3}H]epibatidine suggest that the tested *tert*-butylbispyridinium regioisomers exerted an allosteric effect.

The functional measurements using SSM-based electrophysiology confirmed that MB327 and its regioisomers PTM0001 and PTM0002 are acting as "resensitizers", more precisely type II PAM.

For all three *tert*-butylpyridinum regioisomers can be concluded that the observed nAChR-mediated actions resulted in a therapeutic effect in soman-poisoned rat diaphragm preparations. However, the reduction of the restoring effect at higher concentrations of the tested bispyridinium compounds seems to assume that additionally non-conducting states of the nAChRs are stabilized.

In the end, these results emphasize the necessity to investigate such receptor active compounds with different methods in order to get insight into the interactions with nAChRs on a molecular basis and to translate these effects to a complex organ system.

Nevertheless, further data must be collected for developing meaningful structure-activity relationships.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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6. Zusammenfassung

Bei der Behandlung von Vergiftungen mit phosphororganischen Verbindungen gibt es immer noch therapeutische Lücken. Insbesondere bei Vergiftungen mit Soman oder Tabun kann die gehemmte AChE nicht mehr adäquat reaktiviert werden. Die Hydrolyse des Neurotransmitters Acetylcholin wird nicht mehr katalysiert, so dass es zur Akkumulation dieses Agonisten im synaptischen Spalt und zur Desensitisierung der mAChR und nAChR kommt. Die muskarinisch innervierten Systeme können mit Atropin kompetitiv antagonisiert werden, aber für die direkte Intervention an den nikotinischen Effektorsystemen sind keine Wirkstoffe verfügbar.

Bispyridiniumverbindungen zeigten *in vitro* eine Wiederherstellung der Muskelfunktion und *in vivo* eine Erhöhung der Überlebensrate, ohne dabei die AChE zu reaktivieren. Um aufzuklären, ob der Effekt Rezeptor vermittelt ist und was genau am Rezeptor bewirkt wird, wurden hierfür aussagekräftige Screeningmethoden entwickelt, etabliert und eingesetzt:

- Radioligand-Rezeptor-Bindungsassay
- Bilayer-basierte Elektrophysiologie
- Muskelkraftuntersuchungen am Rattendiaphragma

Für die Untersuchung der Affinität von Liganden an nAChR und mAChR wurden Ligand-Rezeptor-Bindungsassays auf Basis der Filtrationstechnik und Radioisotopen entwickelt. Hierfür wurden Plasmamembranpräparation eingesetzt, die im Fall der humanen mAChR kommerziell erworben, bei humanen α7 nAChR und Muskeltyp-nAChR (*Torpedo californica*) selbst hergestellt wurden.

Um zu erkennen, welchen Effekt die zu testenden Substanzen auf den nAChR ausüben, wurde eine Bilayer-basierte elektrophysiologische Methode entwickelt, die im Gegensatz zu der Patch Clamp-Technik nicht auf ganze Zellen angewiesen ist. Diese SSM-basierte Elektrophysiologie wurde so konzipiert, dass der desensitisierte, pathophysiologische nAChR gezielt herbeigeführt wurde und die Testsubstanzen auf ihre resensitisierende Wirkung, d.h. Eigenschaft als Typ II PAM untersucht wurden.

Auch die Muskelkraftmessungen mittels indirekter elektrischer Feldstimulation stellen ein Modell dar, das die Gegebenheiten einer Nervenkampfstoffvergiftung wiedergeben soll. Bei Soman vergifteten Ratten-Zwerchfellhemisphären wurde der pharmakologische Effekt der drei *tert*-Butyl-Bispyridinium-Regioisomeren MB327, PTM0001 und PTM0002 untersucht. Es stellte sich heraus, dass es klare Korrelationen zwischen den Effekten auf Rezeptor- und Gewebeebene gibt.

Die Kombination dieser drei verschiedenen Screeningmethoden brachte zu Tage, dass positiv allosterische Modulatoren des nAChR therapeutisch von Interesse sind. Hierbei werden nicht orthosterische, sondern allosterische Bindungsstellen adressiert. Insbeson-

dere Typ II PAM, die in der Lage sind, den desensitisierten in einen funktionalen Rezeptorzustand zu überführen (sogenannte „Resensitizer“), könnten zur Therapie von Nervenkampfstoffen eingesetzt werden. Auch ohne Reaktivierung inhibierter AChE (z.B. bei einer Soman-Vergiftung) ist die Wiederherstellung der Muskelkraft möglich; der pharmakologische Effekt ist rezeptorvermittelt. Erste Struktur-Wirkungsbeziehung deuten darauf hin, dass die Alkylkette zwischen den beiden Heteroaromaten kurz, idealerweise ein Propyl-Linker sein soll. Auch das Substitutionsmuster wirkt sich auf die Aktivität aus, wenngleich in etwas geringerem Ausmaß.

Da die Leitsubstanz MB327 und seine Strukturanaloga noch nicht für einen Medikament tauglichen Wirkstoff geeignet sind, muss noch nach weiteren, besseren Wirkstoffen gesucht werden. In einem iterativen Prozess aus virtuellen (Molecular Modeling), präparativen (zielgerichtete Synthesen) und pharmakologischen Methoden werden diese hier vorgestellten Screeningmethoden einen wichtigen Platz einnehmen.

7. Summary

Treatment of nerve agent poisoning still comprises therapeutic gaps. Especially in the case of soman or tabun poisoning, the inhibited AChE cannot be reactivated adequately. The hydrolysis of the neurotransmitter acetylcholine is reduced, resulting in accumulation of this agonist in the synaptic cleft and desensitization of mAChRs and nAChRs. Muscarinic based effector systems allow a competitive antagonism, but drugs for direct intervention at nicotinic acetylcholine receptors are missing.

Bispyridinium compounds showed *in vitro* a recovery of muscle force and *in vivo* increased survival without reactivation of the AChE. However, it was unknown if and which effect was receptor-mediated. For enlightenment of the mechanism and search of more effective substances, screening methods were developed, established and used:

- Radioligand-receptor-binding assay
- Bilayer-based electrophysiology
- Muscle force measurements using rat diaphragm hemispheres

Ligand-receptor binding assays basing on filtration technique and radioisotopes allow the estimation of the affinity of chemical substances toward the mAChRs and nAChRs. The plasma membrane preparations used for this purpose were purchased (in the case of human mAChR) or prepared (in the case of human α7 nAChRs and muscle-type nAChRs (*Torpedo californica*)).

For testing functional effects on the nAChRs, a bilayer-based electrophysiological method (so-called SSM-based electrophysiology) was developed. In contrast to patch clamp techniques, this method depends not on whole cells (“cell-free electrophysiology”). Using SSM-based electrophysiology, the desensitized, pathophysiological receptor state was intentionally induced and type II PAM properties (“resensitizing” effects) of the testing substances evaluated.

Additionally, muscle force measurements using indirect electric field stimulation represent a model for OPC poisoning. The application of substances to soman-poisoned rat diaphragm hemispheres detects restoring effects without reactivation of AChE. Combining the results obtained from binding assays, functional receptor assays and diaphragm tests, clear correlations between receptor- and tissue-based screening methods were observed.

The results obtained from these different screening methods showed that positive allosteric modulators are therapeutically of interest. Allosteric ligands address binding sites distinct from orthosteric binding sites. Especially type II PAM, which are able to recover the activity of desensitized nAChRs, may be beneficial in treatment of OPC poisoning. When nAChRs are resensitized, restoring of muscle force is possible – even without reactivation of inhibited AChE (e.g. soman poisoning).

Initial structure-activity-relations (SAR) implicate that short alkyl spacers between the pyridinium moieties, ideally a propyl chain is advantageous for activity. Additionally, the substitution pattern of the pyridinium moieties influences the manner of activity, albeit to a lesser extent.

The leading structure MB327 and its analogous structures lack drug like properties and are not suitable as antidotes. Consequently, more effective drugs have to be identified. As part of an iterative process of virtual (Molecular Modeling), preparative (target-based synthesis) and pharmacological methods, the described screenings methods will take an essential place.

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