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REVIEW ARTICLE

Acetylcholinesterase Inhibitors and Drugs Acting on Muscarinic Receptors-Potential Crosstalk of Cholinergic Mechanisms During Pharmacological Treatment

Ondrej Soukup[1,2,5,#], Michael Winder[3,#], Uday Kumar Killi[4], Vladimir Wsol[4], Daniel Jun[1,2], Kamil Kuca[2,*] and Gunnar Tobin[3,*]

[1]*Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic;* [2]*Biomedical Research Centre, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic;* [3]*Institute of Neuroscience and Physiology, Department of Pharmacology, the Sahlgrenska Academy at the University of Gothenburg, Sweden;* [4]*Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Hradec Kralove, Czech Republic;* [5]*National Institute of Mental Health, Klecany, Hradec Kralove, Czech Republic*

Abstract: ***Background*:** Pharmaceuticals with targets in the cholinergic transmission have been used for decades and are still fundamental treatments in many diseases and conditions today. Both the transmission and the effects of the somatomotoric and the parasympathetic nervous systems may be targeted by such treatments. Irrespective of the knowledge that the effects of neuronal signalling in the nervous systems may include a number of different receptor subtypes of both the nicotinic and the muscarinic receptors, this complexity is generally overlooked when assessing the mechanisms of action of pharmaceuticals.

***Methods*:** We have search of bibliographic databases for peer-reviewed research literature focused on the cholinergic system. Also, we have taken advantage of our expertise in this field to deduce the conclusions of this study.

***Results*:** Presently, the life cycle of acetylcholine, muscarinic receptors and their effects are reviewed in the major organ systems of the body. Neuronal and non-neuronal sources of acetylcholine are elucidated. Examples of pharmaceuticals, in particular cholinesterase inhibitors, affecting these systems are discussed. The review focuses on salivary glands, the respiratory tract and the lower urinary tract, since the complexity of the interplay of different muscarinic receptor subtypes is of significance for physiological, pharmacological and toxicological effects in these organs.

***Conclusion*:** Most pharmaceuticals targeting muscarinic receptors are employed at such large doses that no selectivity can be expected. However, some differences in the adverse effect profile of muscarinic antagonists may still be explained by the variation of expression of muscarinic receptor subtypes in different organs. However, a complex pattern of interactions between muscarinic receptor subtypes occurs and needs to be considered when searching for selective pharmaceuticals. In the development of new entities for the treatment of for instance pesticide intoxication, the muscarinic receptor selectivity needs to be considered. Reactivators generally have a muscarinic M2 receptor acting profile. Such a blockade may engrave the situation since it may enlarge the effect of the muscarinic M3 receptor effect. This may explain why respiratory arrest is the major cause for deaths by esterase blocking.

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INTRODUCTION

A large number of pharmaceuticals intervene with the cholinergic transmission, most commonly by a direct antagonism on muscarinic receptors [1]. Examples of drugs exerting effects *via* intervention of the muscarinic receptor are pharmaceuticals employed in the treatment of the overactive urinary bladder, in obstructive pulmonary diseases and in the treatment of eye diseases [2]. Also, the muscarinic receptor may be either directly or indirectly targeted in pharmacotherapies of the central nervous system [3]. Despite the general occurrence of a composite muscarinic receptor (muscarinic M1 – M5 receptors) population in the neuronal junction, this has generally been disregarded because the

*Address correspondence to these authors at the Biomedical Research Centre, University Hospital Hradec Kralove, Czech Republic; Tel: +42049583447; E-mail: kamil.kuca@fnhk.cz and Institute of Neuroscience and Physiology, Department of Pharmacology, the Sahlgrenska Academy at the University of Gothenburg, Sweden; Tel: +46317863442; E-mail: gunnar.tobin@pharm.gu.se

#Both authors contributed equally to the paper

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drugs exert almost no selectivity between the muscarinic receptor subtypes [4]. Furthermore, the muscarinic M3 receptor is considered to be the principal receptor subtype mediating the parasympathetic contractile response in smooth muscle tissues. As a consequence, any interactions *via* other muscarinic receptor subtypes in neuronal and neuro-effector junctions have been neglected in the assessment of drug effects.

Pharmaceuticals intervening indirectly with cholinergic transmission, such as in the treatment of Alzheimer's disease (AD), generally act on acetylcholine esterase. In organophosphorus intoxication, such as by pesticides blocking the esterase, antimuscarinic treatment is usually combined with reactivators of the acetylcholine esterase (AChE [5]). However, when examining the mechanism of action of the reactivators it has turned out to be complex [6]. One probable mechanism is an antagonism of acetylcholine effects exerted on muscarinic receptors [6c]. Also, this antimuscarinic mechanism of action of the reactivators shows varying degree of selectivity for muscarinic receptor subtypes [7]. Oximes, for example, bind to and antagonize cholinergic effects preferentially *via* muscarinic M2 receptors [8]. Due to the varying significance of the composite muscarinic receptor population in different organs, the functional implication of intervening at a specific level of the cholinergic transmission may be hard to predict in the whole body [9].

Currently, the cholinergic transmission and the interactions of muscarinic receptors in the synapse and at different levels of the reflex arc are reviewed. Divergent effects of pharmaceuticals due to composite cholinergic mechanisms are addressed from the perspective of functional implications of possible interplays between muscarinic receptor subtypes.

CHOLINERGIC TRANSMISSION

The transmitter acetylcholine is a phylogenetically old substance and almost all living organisms are able to synthesize the compound. The evolvement of the acetylcholine synthesizing systems occurred well ahead of the appearance of living organisms expressing a nervous system [10]. Over the years, the possibility of non-neuronal synthesis of acetylcholine has been overlooked in mammals. However, today two sources of acetylcholine synthesis are recognized – neurons and non-neuronal tissues [11]. The non-neuronal sources of acetylcholine have gained increased interest lately. Originally, non-neuronal release of acetylcholine was ascribed either cells correlated to pathology, *e.g.* cancer cells or immune cells, or tissue lining cells, *e.g.* keratinocytes, urothelial cells, airway epithelial cells or endothelial cells [12]. However, it has been found that also other types of cells may produce acetylcholine. One example is cardiomyocytes, in which the production is thought to be protective against hypertrophic stimuli [13].

Acetylcholine is mainly synthetized from acetyl coenzyme A and choline by the enzyme choline acetyltransferase (ChAT) [14]. ChAT mediates the transfer of an acetyl group from acetyl coenzyme A to choline in the nerve terminal. Neuronal ChAT uses choline that is accessible from the extracellular fluid by a high-affinity choline transporter (ChT). The transmitter is then accumulated in the synaptic vesicles by a vesicular acetylcholine transporter (VAChT). However, the mitochondrial enzyme carnitine acetyltransferase (CarAT) may also contribute to the synthesis of acetylcholine. The ChAT-related enzyme CarAT is expressed in all cells [10]. Besides enhancing the ChAT mediated acetylcholine synthesis by cooperative mechanisms, CarAT may by itself cause acetylcholine synthesis [15]. CarAT seems to have a significant role in cholinergic signalling in some tissues. For example, the measurement of the synthesis of acetylcholine in homogenates of the extensor digitorum longus muscles (in the normally sciatic nerve innervated and in the denervated muscle) revealed half of the acetylcholine to be synthetized by CarAT in the former case and almost all of it in the latter case [16]. However, CarAT may also contribute to acetylcholine synthesis in organs consisting of smooth muscle. Specifically, CarAT is the major ACh-synthesizing enzyme in the urothelium in the urinary bladder [17]. Here, as well as in other non-neuronal cells, the polyspecific organic cation transporter (OCT) has been shown to present choline intracellularly.

Acetylcholine release occurs in a quantal (vesicular) and a non-quantal (non-vesicular) way [18]. The quantal release was first described for the neuromuscular junction, but is typical for neuronal transmission [19]. The basis for quantal release is the exocytosis of acetylcholine containing vesicles in the neuron induced by impulse activity. The membrane depolarization causes voltage-gated Ca^{2+} channels to open and exocytosis of the vesicle content starts within sub-milliseconds after calcium influx. The delay of the release is minimized by a large number of calcium channels being accessible close to the active zone [20]. However, the vesicles docking with the presynaptic membrane of the active zone have to undergo a priming reaction before exocytosis [21]. The anchoring of the vesicle to the membrane and its exocytosis include interactions of a number of proteins (*e.g.*, SNAREs, syntaxin and synaptotagmins), whose activation is Ca^{2+} dependent [22]. At the neuromuscular synapses, the content of an acetylcholine quantum is fairly constant (6000–10000 molecules) [23].

The less well-characterized non-quantal release, on the other hand, may occur independently of impulse activity either in neurons or in non-neuronal tissues and is not restricted to the quantal pulsatile nature of release [19c, 24]. One example of the latter is the urothelial release in the urinary bladder, and further, the OCT seems to be of particular importance in the non-neuronal release of acetylcholine [25, 26]. However, the neuronal non-quantal release may involve ChT as well as VAChT that remains in the plasma membrane after the vesicular fusion [24]. It has been speculated that the ChT may transport choline retrogradely and ACh orthogradely.

Acetylcholine may be hydrolyzed by either of two structurally similar enzymes in the body - acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). In the synaptic cleft, acetylcholine is metabolized by AChE within milliseconds after its release from the nerve, forming choline and acetic acid [27]. In non-neuronal cells both AChE and

BuChE are expressed, while BuChE is particularly obvious in blood plasma, the kidneys and the liver. BuChE catalyzes acetylcholine hydrolysis as well [28]. However, it hydrolyses esters with larger moieties, like butyrylcholine or benzoylcholine, more efficiently. The full significance of BuChE is still unclear, but it probably plays a role in the metabolism of cellular intermediates.

In the periphery, acetylcholine is a classical transmitter in the somatomotoric and autonomic nervous systems. The nicotinergic contraction of skeletal muscle and the muscarinic effects mediated *via* the autonomic nervous system are well known [4b, 9, 29]. In the parasympathetic nervous system, acetylcholine transmits signals both in the ganglia and at the effector cell level. In conditions of excessive discharge of the parasympathetic nervous systems, typical signs of cholinergic effects appear [30]. These signs include salivation, lacrimation, urination, defecation, emesis and vomiting, miosis, bradycardia and bronchial obstruction. Pronounced AChE inhibition causes similar symptoms, but affects the neuromuscular signalling as well [31]. The signs of excessive parasympathetic discharges elucidate the functions that are regulated by the parasympathetic nervous system under physiological conditions.

In contrast to the periphery, acetylcholine primarily acts as a neuromodulator within the central nervous system [32]. Here, acetylcholine modulates a number of vital functions such as cognition, reward and motor control. The effects are exerted by activation of both major groups of cholinergic receptors, muscarinic and nicotinic, which are widely distributed throughout the central nervous system [29, 33]. One major pathway is the cholinergic projection from the nucleus basalis to cortical areas, which is particularly related to attention and memory. In dementia, such as AD, the failure of cholinergic transmission is one important cause for the symptoms.

MUSCARINIC RECEPTORS

Muscarinic receptors occur in five subtypes, which all belong to the family of G protein-coupled receptors [9]. The G protein consists of one α-, β- and γ-subunit. Depending on the primary sequence homology of the α -subunit, the G protein is classified as Gs, Gi/o, Gq or G12 [4b]. The muscarinic receptor subtypes couple differentially to the G proteins, and the subunits of G proteins activate distinct cellular pathways. The inhibitory muscarinic M2 and M4 receptors mainly couple to G i/o, whereas the excitatory muscarinic M1, M3 and M5 receptors most frequently couple to G q/11. The muscarinic M2 and M4 receptors mainly exert inhibitory effects by reducing the activity of adenylate cyclase, prolonging the opening of potassium, non-selective cation and transient receptor potential channels. Muscarinic M1, M3 and M5 receptors increase intracellular calcium by mobilizing phosphoinositides that generate inositol 1,4,5-trisphosphate and 1,2-diacylglycerol.

NICOTINIC RECEPTORS

Nicotinic receptors belong to a family of ligand-gated ion channels, where binding of two molecules of acetylcholine results in a conformation change of the receptor and the subsequent formation of a pore between the subunits allows the permeation of cations through the receptor. Usually, sodium (Na^+) ions flow inward and potassium (K^+) outward, however, some neuronal nicotinic receptors have been shown to be permeable for calcium (Ca^{2+}) ions, thereby affecting the release of other neurotransmitters [34]. Two types of nicotinic receptors occur – the muscular and the neuronal type. The muscle-type receptors are situated in the neuromuscular junctions and are composed of α_1, β_1, δ, ε or γ subunits. The adult and fetal isoforms differ by substitution of the γ subunit with the ε subunit. The stoichiometry of the muscle-type nicotinic receptor is always $\alpha_2\beta(\varepsilon)\gamma\delta$. The neuronal type, sometimes subdivided into a CNS type and a ganglionic type, is always composed of α- and β-subunits. Homomeric receptors consist of only $\alpha_{7\text{-}10}$ subunits, heteromeric of $\alpha_{2\text{-}6}$ and β_2.4 subunits in a conserved 2:3 ratio [35].

Nicotinic receptors are localized both pre- and post-synaptically. The former ones serve as regulatory receptors modulating the release of acetylcholine and other neurotransmitters in the CNS. The nicotinic-evoked release of transmitter can be both positively and negatively modulated by interactions with metabotropic and ionotropic receptors [36]. At the motor neurons, presynaptic nicotinic receptors facilitate mobilization of a reserve pool of acetylcholine, thereby enhancing the cholinergic transmission [37]. The significance of postsynaptic nicotinic receptors is evident as they depolarise the membrane at the neuro-muscular endplate, leading to muscle contraction. Further, in the CNS they play a crucial role in maintaining cognitive function. Nicotinic receptors in the CNS are commonly linked to neurodegenerative diseases such as Alzheimer´s and Parkinson's disease [38].

SALIVARY GLANDS

The increase in salivary flow evoked by activation of muscarinic receptors has generally been attributed to effects *via* muscarinic M3 receptors [39]. However, all subtypes have been described in salivary glands [40] and data from studies in several animal models have indicated that in particular muscarinic M1 receptors also contribute to the secretory response to muscarinic receptor stimulation [41]. In the rat sublingual and the ovine submandibular glands, concomitant activation of the different muscarinic receptor subtypes seems to be a necessity for maximum glandular responses [41a, 42].

Regarding the neuronal activation of salivary glands, prejunctional muscarinic receptors modulate the parasym-pathetic nerve-evoked response [43]. Here, muscarinic M1 receptors normally facilitate transmitter release during short, intense nerve activity. At low frequencies, on the other hand, muscarinic M2 receptors, or possibly muscarinic M4 receptors, inhibit the transmission, but only after some delay [42, 43b]. By blocking the prejunctional inhibitory muscarinic receptors the nerve-evoked secretory response may be threefold larger than in the absence of the inhibition in the rat parotid gland [43b]. This increase, however, does not only reflect cholinergic effects since neuropeptides, *e.g.*, vasoactive intestinal peptide (VIP), are co-released from the

parasympathetic nerve and markedly potentiate the cholinergic response. Also, in many species the neuropeptides evoke atropine-resistant overt secretion (up to 50% of the parasympathetic response) [44]. In the concomitant parasympathetic vascular response, acetylcholine and VIP also interact [45]. Here, VIP is the major player, while acetylcholine, by acting on muscarinic M3 receptors, has additive effects [46].

The Urinary Bladder

In the urinary bladder, all five subtypes of the muscarinic receptor are expressed [47]. However, the expression varies in different bladder tissues. On the detrusor smooth muscle cells, muscarinic M2 receptors dominate in number, even though muscarinic M3 receptors have been shown to evoke the main cholinergic contractile response [43a, 48]. The other subtypes have been demonstrated to occur as well, but at a markedly lower number. In the urothelium, on the other hand, all muscarinic receptor subtypes are expressed in large numbers. The muscarinic M1 receptors occur on basal cells and muscarinic M2 receptors on umbrella cells, while muscarinic M3 and M4 receptors are homogenously distributed and muscarinic M5 receptors are distributed with a decreasing gradient from luminal to basal cells [49]. Traditionally, the urothelium has been regarded to be a passive barrier. However, during the last decade observations have accrued showing that the urothelium plays an important integrating role in the regulation of bladder function [50]. This indicates that the ways the muscarinic receptors may interact are numerous, which may be even more intricate when taking prejunctional receptors into consideration. Namely, muscarinic receptors are expressed on bladder neurons, afferent as well as efferent [43a, 51]. Muscarinic facilitatory and inhibitory receptors occur on efferent nerve terminals. While the former are of the muscarinic M1 receptor subtype, the latter has for long been considered to be of the M2 subtype. However, some reports indicate that the inhibitory subtype may be the muscarinic M4 receptor [47, 52]. Sensory neurons have been reported to express different subtypes of the muscarinic receptor (M2, M3 and M4 [53]), suggesting cholinergic influence on the processing of sensory information from the bladder. However, even though it is well known that the administration of muscarinic antagonists inhibits the neuronal activity in bladder afferents [54], the specific effects of the muscarinic receptor subtypes in the local bladder afferent activation are unclear.

A number of substances may be released from the urothelium of which ATP seems to be of particular interest [55]. Stretch may release urothelial ATP that, directly or indirectly *via* inducing non-neuronal acetylcholine release, stimulates afferents within the micturition reflex [56]. Non-neuronal acetylcholine may, by acting on urothelial muscarinic M5 receptors, induce the release of nitric oxide that exerts inhibitory effects and that preserves low bladder pressures in spite of increasing urine volumes [57]. Any muscarinic M3 receptor effect on the effector smooth muscle cell evoked by low concentrations of acetylcholine, *e.g*. non-quantal release or release at low parasympathetic stimulation intensity, is counteracted by muscarinic M2 receptor hyperpolarization [58]. Also, muscarinic M2 receptors inhibit adenylate cyclase and by that the formation of cAMP [59]. This mechanism has been shown to prevent adrenergic and purinergic effects that might counteract the cholinergic contraction. Furthermore, the release of low amounts of acetylcholine upon parasympathetic activity exerts a prejunctional inhibition on its own release *via* muscarinic M4 (and/or M2) receptors. However, intense neuronal activity over short periods of time resulting in high concentrations of the transmitter stimulates, in resemblance with the glandular mechanism, facilitatory muscarinic M1 receptors on the nerve terminal [41c, 43b]. This eventually results in either larger (M1 effect) or smaller (M4 (and/or M2) effect) acetylcholine evoked detrusor contractions *via* the muscarinic M3 receptor. A schematic overview of the conceptual interactions of muscarinic receptor subtypes within the urinary bladder is presented in Fig. (**1**).

THE RESPIRATORY TRACT

The respiratory tract shows great resemblance with the urinary bladder regarding muscarinic receptors and the cholinergic system. All five subtypes are expressed in the lungs and both neuronal and non-neuronal release of acetylcholine occurs [60]. Muscarinic M3 receptors have a dominant role in the smooth muscle contraction. Furthermore, the muscarinic M2 receptors that outnumber the M3 receptors have indirect roles. These indirect roles are exerted by inhibition of relaxation evoked by β-adrenoceptors [61]. Both facilitatory and inhibitory prejunctional muscarinic receptors regulate the parasympathetic transmission [62]. In resemblance with the urinary bladder and salivary glands, prejunctional muscarinic M1 receptors facilitate the acetylcholine release [63]. However, most reports claim the inhibitory receptors to be of the M2 subtype, even though some animal studies propose the prejunctional receptors to be of the M4 subtype [64]. Nevertheless, the prejunctional inhibitory muscarinic receptor function has been suggested to be hampered in asthma [62]. In addition to the smooth muscle expression of muscarinic receptors, mucosal glands also exhibit muscarinic receptors and likewise the M3 subtype mediates the main secretory response [65].

The pulmonary arteries belong to the group of vessels, including those of salivary glands as well, that are influenced by parasympathetic nerve activity [66]. Namely, in the lung, stimulation of the vagal nerve induces vasodilatation *via* acetylcholine acting on muscarinic M3 receptors [67]. This may in turn cause endothelial release of nitric oxide [68]. The vascular response to acetylcholine appears biphasic, involving a contractile part as well. Muscarinic M1 receptors have been discussed in this context and have been proposed to have a key role in pulmonary vascular dysfunction [69]. However, the vagal transmitter release is not the only source of acetylcholine [70]. Non-neuronal release of acetylcholine from the endothelium has been proposed to participate in the vasodilator response due to local mechanical forces induced by blood flow changes [12].

THE HEART

The primary effect of the parasympathetic nervous system in the heart is to decrease the rate [71]. The source of acetylcholine release may be an atrial non-neuronal

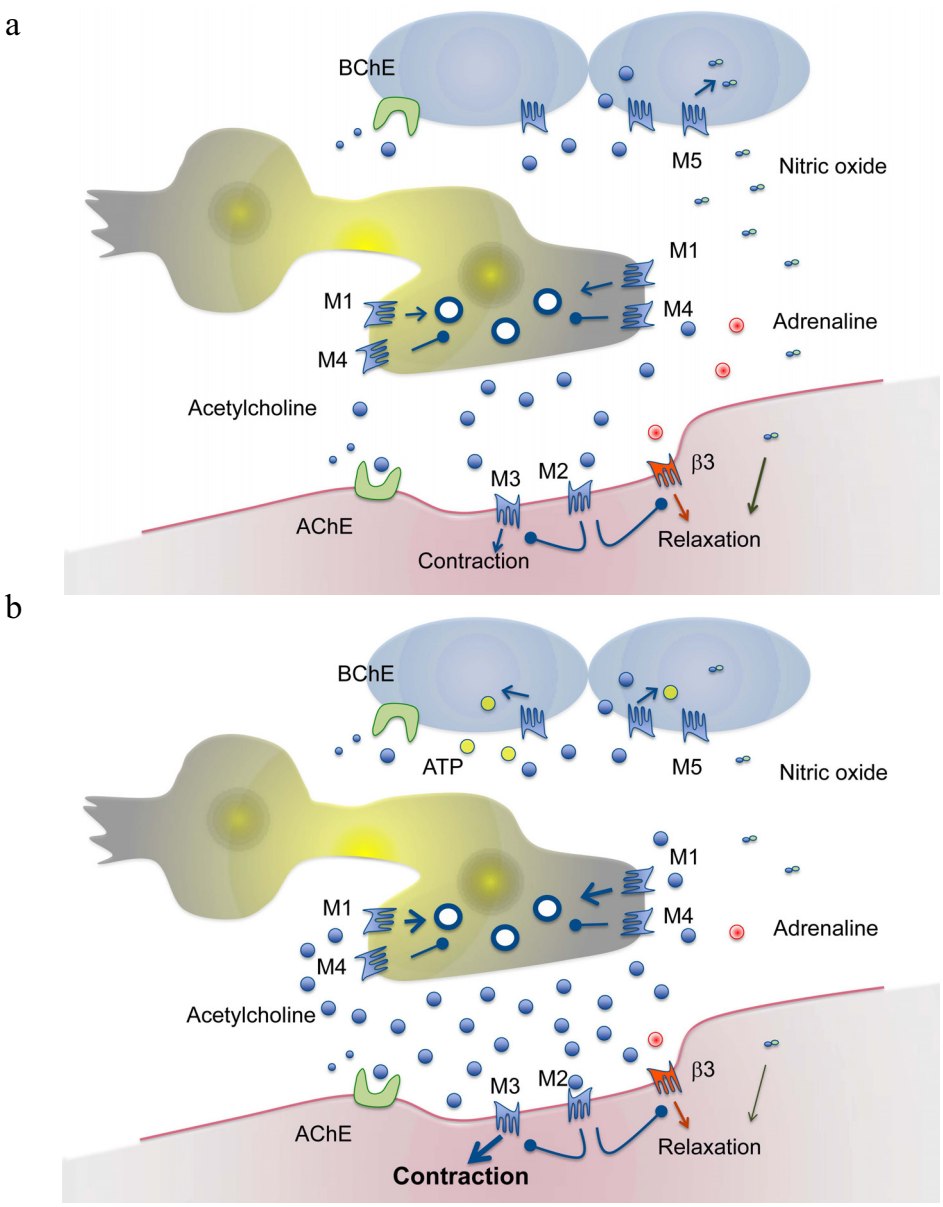

Fig. (1). Schematic drawing of muscarinic receptor subtypes in the urinary bladder. The upper panel (**a**) indicates the filling phase of the urinary bladder. In this phase a limited amount of acetylcholine is released from neuronal (parasympathetic) and non-neuronal tissues (*e.g.* the urothelium). Adrenergic stimulation of relaxatory β3-adrenoceptors (by noradrenaline or adrenaline) is indicated. Relaxatory urothelial factors (*e.g.* nitric oxide released by activation of muscarinic receptors) stabilize the detrusor. Postsynaptic muscarinic M2 receptors inhibit the effects of contractile muscarinic M3 receptors. Presynaptic muscarinic M4 receptors inhibit the release of transmitter. The lower panel (**b**) indicates the emptying phase (detrusor contraction). In this phase a large number of vesicles release acetylcholine. Presynaptic muscarinic M1 receptors are activated and facilitate the release. The intense stimulation of contractile muscarinic M3 receptors overcomes any inhibitory effect of postsynaptic muscarinic M2 receptors. The muscarinic M2 receptors inhibit relaxatory effects *via* β3-adrenoceptors. Excitatory factors (*e.g.* ATP) are released from the urothelium. Both synaptic acetylcholinesterase and non-synaptic butyrylcholinesterase metabolizes acetylcholine.

production [18a]. The classical view is that atrial muscarinic M2 receptors exclusively mediate this effect [72]. However, studies in a number of different species including man, indicate the expression of other subtypes, in particular in the ventricles [73]. Of these other subtypes, most interest has been given to muscarinic M1 and M3 receptors [74]. Whether functionally significant or not, all other subtypes are heavily outnumbered by the muscarinic M2 receptors [75].

Cholinergic stimulation of the atrial muscarinic M2 receptors slows the rate of spontaneous firing in the sinoatrial node [76]. Also, muscarinic M2 receptors influence the conductance of impulses in the atrioventricular node, which is slowed down by parasympathetic activity [77]. One way this effect of muscarinic M2 receptors is exerted is *via* potassium currents ($I_{K(ACh)}$) that hyperpolarizes the membrane potential [76]. Another way is by inhibiting cAMP-dependent effects (*i.e.*, β-adrenoceptor effects [78]).

Several mechanisms have been suggested regarding the latter matter. For instance, muscarinic M2 receptors have been suggested to stimulate the production of cGMP *via* nitric oxide, which in turn inhibits cAMP-dependent responses.

In the heart, large concentrations of muscarinic agonists have been shown to increase automaticity and possibly contractility as well [74b, 79]. Muscarinic M1 and/or M3 receptors have been discussed in this context. A muscarinic M1 receptor effect has been suggested to be mediated *via* PLC-dependent mechanisms that enhance L-type Ca^{2+}-channels [80]. The tentative muscarinic M3 receptor effect seems also to include PLC-dependent mechanisms, which activate potassium currents of a delayed rectifier type [81]. Thus, the most prominent and normal role of acetylcholine in the heart is to decrease the cardiac output *via* muscarinic M2 receptors. However, other subtypes may also affect cardiac function, particularly under pathological conditions such as in heart failure, ischemia and arrhythmias [82].

THE CENTRAL NERVOUS SYSTEM

All five muscarinic receptor subtypes occur in the central nervous system. In contrast to the periphery, based on their frequency of occurrence, muscarinic M3 receptors seem to be of less significance [83]. Muscarinic M3 receptors are mainly expressed in cortical pyramidal cells and in glial cells. Muscarinic M1 receptors show a similar distribution and are found throughout the brain, but in much higher concentrations [84]. The highest concentrations occur in cortical regions and hippocampus. The cortical muscarinic M1 receptors are mostly located postsynaptically in excitatory synapses. Muscarinic M2 receptors occur both pre- and postsynaptically, and the receptor subtype occurs in high levels in the nucleus basalis, cerebellum, pons/medulla and thalamus/hypothalamus [85]. Muscarinic M2 receptors are expressed in other cortical areas also, such as hippocampus and putamen. Muscarinic M4 receptors occur in the highest concentrations in basal ganglia, where they occur in association with dopaminergic receptors. Muscarinic M5 receptors, on the other hand, are sparsely expressed in the central nervous system [86]. However, some expression occurs in the hippocampus, substantia nigra and ventral tegmental area.

The cholinergic systems are affected in a number of disorders of the central nervous system. In particular in AD and other cognitive disorders, muscarinic receptors have been regarded to be potential targets when developing new pharmaceuticals. Muscarinic M1 receptors seem to be particularly important for mediating cognitive effects, and M1-selective agonists have been proposed to be suitable in the pharmacotherapy of AD. The favourable effects of treatment with acetylcholine esterase inhibitors support this notion.

APPLICATION OF PHARMACEUTICALS ACTING ON THE PARASYMPATHETIC NERVOUS SYSTEM

A number of pharmaceuticals used in the clinic intervene with the cholinergic systems and because of the broad spectrum of parasympathetic effects in the body, adverse effects are numerous [1]. Historically, drugs affecting the cholinergic systems were often employed in the pharmacotherapy of diseases in the gastrointestinal tract *e.g*. gastric ulcer and intestine disorders [87]. Today, other treatments have substantially replaced the old therapies. However, the use of pharmaceuticals acting on the parasympathetic nervous system is still indicated in the pharmacotherapy, but then non-systemic administration approaches of the drugs may be employed [88].

Generally, systemic administration of muscarinic receptor antagonists is more common than of muscarinic receptor agonists. When agonists are used the indication often only requires a single dose administration. In cases of atonic gut or atonic bladder, a muscarinic agonist may be injected in order to trigger the activity of the smooth muscle [89]. However, in the treatment of glaucoma (local) or oral dryness as in Sjögren's syndrome (systemic) pilocarpine may be prescribed for long-term use [90]. Acetylcholine esterase inhibitors are an alternative to the direct agonists and are indicated in the treatment of diseases in the central nervous system (*e.g*. AD; see below [91]). Outside of the central nervous system they may be used in the treatment of glaucoma, to increase bladder and intestine motility, to overcome neuromuscular blockade in anaesthetized patients and in the treatment of myasthenia gravis. Esterase inhibitors with strong and irreversible binding to the enzyme have been used in chemical warfare or misused for terroristic purposes. Also, pesticides have the same mechanism of action as the inhibitors used in warfare and may cause, likewise to the militarily used inhibitors, death mainly by respiratory arrest. In the treatment of esterase inhibition, so called esterase reactivators are given in addition to muscarinic antagonists [92]. The mechanism of action of the reactivators is *via* affinity to the enzyme and the ability to bind the organophosphate which is inhibiting its active site, thereby making the site accessible to acetylcholine [93]. In the absence of inhibition, the reactivators act as weak acetylcholine esterase inhibitors. The exact mechanism of action of the reactivators is not totally unraveled. However, muscarinic and nicotinic receptor antagonism has been suggested [92].

Even though muscarinic receptor antagonists may be administered locally, *e.g*. for inducing mydriasis, counteracting cycloplegia and as asthma inhalation treatment [88, 90b], they are applied systemically in the treatment of certain diseases and conditions such as for resuscitation purposes *via* inhibition of bradycardia and for relief of postprandial discomfort [82, 87, 94]. However, the most common use of drugs exerting antimuscarinic receptor effects is for urge incontinence. Also, in cases of organophosphate poisoning (chemical warfare or pesticides), muscarinic receptor antagonists are indicated as antidotes [92].

TREATMENTS WITH ANTICHOLINERGIC DRUGS AFFECTING THE LOWER URINARY TRACT

Today it is well accepted that bladder contraction is almost solely caused by activation of muscarinic M3-receptors, with slight influence of M2-receptors *via* indirect inhibition of adrenergic and purinergic induced detrusor relaxation [47]. However, when employing anticholinergic drugs in the treatment of the patient, the whole micturition reflex arc must be taken into consideration. Even though the

rationale for anticholinergic treatment of overactive bladder is *via* blockade of muscarinic receptors, the effect is likely exerted at more levels than merely by antagonizing receptors in the detrusor [95]. For instance, one must take non-neuronally released acetylcholine into account, which can act on muscarinic M2, M3 and M4 receptors, yielding an excitatory or inhibitory effect on afferent activity in the micturition arc. An anticholinergic effect on such excitatory muscarinic receptors is suggested to at least partially improve the disease symptoms. When examining the affinity of the antimuscarinic drugs, most of them have greatest affinity for muscarinic M3 receptors with some exceptions such as imidafenacin and tolterodine [96]. Therefore, if one solely takes into account the detrusor overactivity in OAB patients to be caused by activation of muscarinic M3 receptors located either on the detrusor muscle [97] or on afferents [98], it is impossible to explain the positive clinical effects of the drugs.

Over the past decade and a half, the concept of tissue selectivity has emerged which could be one possible approach to develop new entities showing less anticholinergic adverse effects. A number of studies have indicated that some antagonists exert this form of bladder selectivity, in comparison with salivary glands when examined *in vivo* [99]. The suggestion that these observations show general bladder selectivity may be an over-interpretation. No, or only small, differences appear when studying the tissue affinity of antagonists when comparing the bladder and salivary glands (Table **1a**). The low degree of selectivity is also supported by results from binding experiments on cell lines expressing the specific muscarinic receptor subtypes (Table **1b**). Data from one species must be assessed based on its particular physiology. In rat a pronounced non-adrenergic, non-cholinergic (NANC) salivary secretion occurs, while in cat the NANC transmitters (*e.g.*, VIP) markedly potentiate the cholinergic response [9]. No such responses have been described in man. It may be argued that the same situation is valid in the urinary bladder. However, the NANC transmitters are different [100]. In the case of the occurrence of NANC responses, ATP (short-lasting effects) is the main transmitter in the bladder [101], while neuropeptides (substance P, VIP and calcitonin gene-related peptide; long-lasting effects) evoke the atropine-resistant responses in glands [102]. Therefore, it is hazardous stating tissue selectivity based on responses evoked by nerve stimulation. Also, carbachol has routinely been used as the muscarinic agonist of choice in studies on the urinary bladder. This may, likewise to acetylcholine, also create a confounding situation due to its pronounced effect on nicotinic receptors [103]. Thus, to conclude the occurrence of tissue/bladder selectivity, *in vivo* data could be faulty for various reasons. For one, bladder

Table 1a. Inhibitory constants of muscarinic antagonists in urinary bladder and salivary gland tissue.

Compound	Urinary Bladder (pKi)	Parotid Gland (pKi)	Submandibular Gland (pKi)
Imidafenacin	9.2[a]	9.2[a]	
Tolterodine	8.6[a], 8.7[b], 8.5[c]	8.5[a], 8.5[b]	8.7[c]
Oxybutynin	8.4[a], 7.8[b], 8.7[c]	8.8[a], 8.2[b]	9.0[c]
Solifenacin	7.4[a], 8.5[c]	7.7[a]	8.2[c]
Darifenacin	8.4[c], 7.7[b]	8.7[b]	8.8[c]
Propiverine	6.3[b]	6.6[b]	

Competitive inhibition of [b][^{3}H]NMS [149] and [a][^{3}H]imidafenacin [96d] binding by muscarinic antagonists in homogenates of human bladder and parotid gland and [c] of carbachol-induced Ca^{2+} mobilization in bladder and submandibular gland cells from Cynomolgus monkeys [150].

Table 1b. Binding affinities of muscarinic antagonists in cell lines expressing human muscarinic receptor subtypes.

Compound	M1 (pKi)	M2 (pKi)	M3 (pKi)	M4 (pKi)	M5 (pKi)
Tolterodine	8.7,[c] 7.8[b]	8.4[c], 8.0[b]	8.4[c], 8.3[b]	8.4[c], 8.6[b]	8.8[c], 8.6[b]
Oxybutynin	8.6[c], 8.5[a]	8.1[c], 7.6[a]	8.8[c], 8.7[a]	8.7[c], 8.5[a]	8.8[c]
Solifenacin	7.6[c], 7.1[b]	7.1[c], 7.0[b]	7.7[c], 7.7[b]	6.8[c], 7.7[b]	7.2[c], 7.5[b]
Darifenacin	7.5[c], 7.2[b]	7.2[c], 6.9[b]	8.6[c], 8.4[b]	7.3[c], 7.5[b]	7.9[c], 7.6[b]
Fesoterodine	7.8[b]	8.6[b]	7.3[b]	8.0[b]	
Propiverine	6.4[a]	5.7[a]	6.7[a]	6.5[a]	
Trospium	8.5[b]	9.0[b]	9.0[b]	8.8[b]	8.2[b]

References:
a=151 b=152 c=153

contraction and salivation are induced in different ways. This, in turn, is linked to the possibility that other factors may obscure the data and its interpretation. For instance, it seems likely that effects involving prejunctional inhibitory muscarinic receptors, *i.e*. M2 and/or M4, will affect measurements of salivation and bladder contraction [43, 52, 60, 64b, 104]. Further, it is still not known exactly how the various muscarinic receptor subtypes *per se* affect bladder contraction and salivation in different species, and to link findings in an animal species to possible effects in man is therefore hard. However, the fact remains that *in vivo* data of this kind are hard to interpret due the influence of prejunctional receptors and a non-complete understanding of bladder contraction in other species, as well as the various levels at which antimuscarinics affect bladder contraction.

TREATMENTS WITH ANTICHOLINERGIC DRUGS AFFECTING THE RESPIRATORY TRACT

Anticholinergic drugs are often employed in the treatment of chronic obstructive pulmonary diseases in which an increased vagal tone may occur [105]. Consequently, the drugs may exert a number of attractive effects, which in addition to counteracting the smooth muscle contraction, decreases mucus secretion and bronchial vasodilatation [106]. However, in cases of the anticholinergic drug reaching the circulation, adverse effects such as dry mouth, urine retention and tachycardia often occur. Anticholinergics may also be indicated in asthma therapy when the condition is associated with vagal activity. However, β_2-agonists are generally superior in improving airflow in acute asthma when compared with anticholinergics [107].

Two types of muscarinic receptor antagonists are approved for marketing authorization; so called short-acting (*e.g*., ipratropium and oxitropium) and long-acting muscarinic receptor antagonists (*e.g*., tiotropium) [108]. Even though tiotropium is more potent than ipratropium as an antagonist at muscarinic receptors, its longer dissociation half-life at muscarinic M3 receptors provides the long-acting duration.

A number of new anticholinergic drugs are currently in phase II or III. One mutual focus when new anticholinergic drugs are developed is to create compounds with larger effects on muscarinic M3 receptors than on muscarinic M2 receptors [109]. This aims to reduce the cardiac adverse effects induced by the treatment. Namely, increased risks of cardiovascular death have been reported [110]. Furthermore, another positive result of this new selectivity profile could be that less effect occurs on the prejunctional inhibitory muscarinic receptors. However, it is still not clarified if these latter receptors are of the muscarinic M2 or the M4 receptor subtype in humans, even though animal experiments indicate the receptors to be of the latter subtype [111]. If they are of the muscarinic M4 receptor subtype, selectivity for muscarinic M3 over M2 receptors might be of no significance regarding the prejunctional effects. Furthermore, the selectivity for muscarinic M3 over M2 receptors may even be disadvantageous in view of the muscarinic M2 receptor inhibiting adrenergic relaxation [112]. Namely, if only the muscarinic M3 receptors are blocked, while leaving the muscarinic M2 receptors unaffected, acetylcholine can still exert its M2-mediated inhibition of β_2-adrenoceptor induced relaxation.

TREATMENTS WITH PHARMACEUTICALS AFFECTING ACHE

As have been mentioned previously, AChE modulators can be used in the clinical treatment of several conditions. AChE modulators comprise of two different subgroups - widely used AChE inhibitors and specific AChE reactivators. One important characteristic of the latter subgroup is that they usually act as weak AChE inhibitors [7]. However, the specific name originates from the pharmacological action, which is reactivation and which is ensured by the oxime moiety in their structure. Therefore, the term oxime reactivator, or just plainly oxime, is commonly used. Both types of compounds affecting the esterase show composite mechanisms of action. A summary of AChE affecting compounds is presented in Table **2**. While the AChE inhibition frequently is combined with effects on the nicotinic receptor, the effect on muscarinic receptors is particularly obvious for the reactivators.

REACTIVATORS OF ACHE

Reactivators are used in a specific case - the organophosporus (OP) poisoning caused by either nerve agents like soman, sarin tabun *etc*. or by OP pesticides [92]. The former cause is nowadays rather rare, however, recent misuse of sarin in Syria testifies in favor of maintaining readiness against OP terroristic threats. Moreover, their place in the army gear is unquestionable. Reactivators, together with atropine, represent a necessary first aid for OP intoxication. Every year, hundreds of thousands of people die due to pesticide poisoning, either by mistake or rather commonly by committing suicide [113]. Reactivators represent the causal treatment whereas atropine is merely symptomatic [114]. Pralidoxime, trimedoxime, methoxime, obidoxime and HI-6 are reactivators that are currently used against OP poisoning. Unfortunately, the reactivators differ in their efficacy against individual nerve agents, which means that the source of poisoning must be identified before application of a proper oxime. The most versatile oxime, HI-6, lacks efficacy against tabun for which obidoxime or trimedoxime must be used. On the contrary, those compounds are ineffective against soman poisoning (for review see [115]). Soman poisoning is also complicated due to the process called “aging”, which is a time-dependent loss of AChE ability to be reactivated [116]. Regarding soman, aging occurs within a few minutes. Pesticide poisoning is easier to treat, since aging may take a couple of days. Obidoxime is the drug of choice here. However, due to its higher toxicity, a replacement of obidoxime would be beneficial and the oxime K027 seems to be a rather promising candidate [117]. Reactivators also exhibit varying degree of affinity for cholinergic receptors [7]. Obidoxime, for instance, exerts significant antagonism on the muscarinic M2 receptor [8]. At first, this mechanism of protecting cholinergic receptors may seem advantageous. However, in the light of what has been discussed above regarding the physiological function of this particular receptor, it may explain its higher toxicity [58]. Another common feature of

Table 2. IC_{50} values (in μM) representing affinity of different AChE inhibitors to cholinergic enzymes (human acetylcholinesterase (hAChE) and human butyrylcholinesterase (hBAChE), cholinergic receptors (muscarinic; Musc) and nicotinic (Nic) receptors.

Compound	hAChE	hBChE	Musc	Nic
Pralidoxime	>1000[a]	>1000[b]	N/A	N/A
Trimedoxime	215[a]	>1000[b]	N/A	N/A
Methoxime	>1000[a]	>1000[b]	N/A	N/A
Obidoxime	>1000[a]	>1000[b]	3.5[i]	140-250[j]
HI-6	281[a]	>1000[b]	167[i]	140-250[j]
K027	>1000[a]	>1000[b]	18[i]	>600[j]
K203	>1000[a]	>1000[b]	4.7[i]	140-250[j]
Physostigmine	0.072[g]	0.035[g]	24000[q]	Direct agonism[s] Potentiation(1-100 μM)[p] Antagonism (IC_{50}=70 μM)[s]
Pyridostigmine	40[c]	16000[c]	>10[v]	Potentiation(200-400 μM)[x] Antagonism (IC_{50}=2000 μM)[x]
Neostigmine	0.1[c]	0.8[c]	No affinity[r,v]	100[t]
Ambenonium	0.0007[c]	6.82[c]	0.37[u]	Direct agonism[w]
Distigmine	0.25[g]	0.27[g]	Ki=2[u]	Ki=23[u]
Edrophonium	5.17[c]	1370[c]	>10[v]	Direct agonism (<300 μM][k] IC_{50}=44[k]
Tacrine	0.5[d]	0.02[d]	12.6[d]	3.6[d]
Donepezil	0.022[e]	4.15[e]	21[m]	Antagonism (10-100 μM)[n]
Rivastigmine	4.15[f]	0.037[f]	No affinity[o]	15-30[p]
Galantamine	0.8[f]	73[f]	>10[v]	Potentiation (1-10 μM) Antagonism (>10 μM)[l]

References:
a=[154] b=[155] c=[156] d=[157] e=[158] f=[159] g=[160] h=[161] i=[162] j=[163] k=[164] l=[165] m=[166] n=[167] o=[168] p=[169] q=[170] r=[171] s=[172] t=[173] u=[147] v=[174], w=[175] x=[176].

all reactivators is a low blood-brain barrier penetration due to the charged nitrogen in their structure [114b]. Therefore, the use of non-quaternary and monoquaternary reactivators or other approaches targeting blood-brain barrier penetration are currently being thoroughly investigated. An oxime moiety seems to be essential for the effects of reactivators. Attempts with compounds lacking this moiety have been made, but these compounds are not better than oxime-based reactivators regarding efficacy against OP compounds (for review see [118].

ACETYLCHOLINESTERASE INHIBITORS

Inhibitors of AChE have found their use in a wide area of applications. Reversible inhibitors can serve as prophylaxis for OP poisoning. The resistance to OP irreversible inhibitors is ensured by their higher affinity to AChE than organophosphate, and after being spontaneously hydrolyzed AChE can fulfill its physiological tasks. Carbamates (pyridostigmine, physostigmine) belong to this group of reversible AChE inhibitors [114a].

The use of AChE inhibitors is wide, for instance as symptomatic treatment in Alzheimer´s disease (AD) and other types of dementia (*e.g*. Lewy body dementia), glaucoma, postural tachycardia and myasthenia gravis [119]. Especially AD represents an increasing health problem [120], and the massive worldwide pursuit for finding an effective cure has been fruitless so far. In the treatment of AD, the fact that inhibitors of AChE might also be inhibitors of BuChE should be considered. It has been reported that non-selective inhibitors of cholinesterases would be a more nuanced and logical treatment, since BuChE activity rises in the AD brain [121]. As expected, selective AChE inhibitors have been reported to be more effective than inhibitors of BuChE due to the fact that AChE outnumbers BuChE in the brain [119a]. Since one area of frequent employment of cholinesterase inhibitors is AD, the pharmacology of some key inhibitors are reviewed with this particular focus.

The first nonselective AChE inhibitor was tacrine (1,2,3,4-tetrahydro-9-aminoacridine), commercially named (Cognex®), which was approved by the FDA in 1993.

However, the use of tacrine was limited by its poor oral bioavailability, the necessity of four-times daily dosing, and considerable adverse effects (mainly hepatotoxicity) [122]. Due to the adverse effects, the compound was eventually withdrawn from the market. Interestingly, tacrine is a relatively weak AChE inhibitor, as compared to donepezil or rivastigmine, with a complex mechanism of action involving other cholinergic structures, ion channels and monoaminergic systems [123]. Furthermore, tacrine reduces the levels of total β-amyloid peptide (Aβ), suggesting its involvement in the amyloidogenic pathway [124]. Even though tacrine itself is an obsolete molecule, it is a parent compound for many derivatives due to its multi-target directed ligand (MTDL) mode of action. Such compounds have recently been reported to combine inhibition of cholinesterases with antioxidant activity, chelating of metal ions, interaction with cholinergic receptors, neuroprotection and the inhibition of Aβ turnover [125].

Later, rivastigmine (Excelon®), another non-selective cholinesterase inhibitor, was approved for the treatment of mild to moderate AD. Rivastigmine is a pseudo-irreversible selective AChE inhibitor [126]. The pseudo-irreversibility refers to its binding to AChE, which cleaves the rivastigmine molecule resulting in the release of an inert phenolic product while the carbamate moiety remains bound to the esteratic site of the AChE [127]. Similarly to tacrine, rivastigmine seems to possess additional mechanisms of action of importance in the treatment of AD, even though probably not to same extent. Nevertheless, it has been reported to beneficially modulate the glutaminergic pathway [128] and shift Aβ processing towards the more beneficial α-secretase cleavage pathway [129]. The latter effect on the Aβ cascade might be a common feature of all BChE inhibitors since the locally neurotoxic Aβ plaque development is driven by the increase of BChE at the plaque level [128].

Galantamine, an alkaloid from the family *Amaryliidacae*, represents the weakest AChE inhibitor among the approved compounds. However, it also has additional cholinergic effects. Galantamine acts as a positive allosteric modulator on nicotinic receptors in the central nervous system, which are down regulated in AD patients [130]. Besides the direct and indirect cholinomimetic effect, galantamine modulates the nicotinic receptor-induced release of glutamate and monoamines, such as serotonin and norepinephrine, which also play a role in the pathogenesis of AD [131].

The last, but probably most widely used and most effective, AChE inhibitor is donepezil. Its efficacy is ensured by its simultaneous binding to the active site and to the peripheral anionic site of the enzyme [132], which has been reported to enhance Aβ aggregation [133]. Thus, donepezil stimulates the cholinergic pathway and inhibits Aβ turnover in the AD brain.

Despite the variations in the mode of action of the three cholinesterase inhibitors, which do notonly pertain AChE inhibition, there is no evidence of any major differences between them in respect to efficacy against AD. However, there appears to be less adverse effects associated with donepezil [134]. Newly developed derivatives of currently used AChE inhibitors have been reviewed recently [135].

Another disease in which AChE inhibitors are prescribed is myasthenia gravis. Myasthenia gravis is a rare disease affecting 20 out of 100 000 people [119b, 136]. Since the target is the motoric synapse at the neuromuscular junction, the AChE inhibitors of choice, in contrary to those used in the treatment of AD, are charged molecules. The anticipated clinical effect is simply an enhancement of cholinergic transmission, which is defected by an antibody-dependent attack on the postsynaptic membrane in the neuromuscular junction [137]. Antibodies may either affect the muscular nicotinic receptor (seropositive myasthenia gravis) or other components in the neuromuscular junction (seronegative myasthenia gravis) [138]. It has been shown that many seronegative patients have antibodies that bind to the muscle-specific tyrosine kinase that anchors and clusters the nicotinic receptor at the postsynaptic membrane [139].

Similarly to the treatment of AD, tAChE inhibitors employed in the treatment of myasthenia gravis only have symptomatic effects, by increasing acetylcholine and thereby facilitating neuromuscular transmission. The carbamates neostigmine, pyridostigmine and the extremely effective ambenonium are commonly used drugs [140]. Furthermore, edrophonium is a rapidly acting and reversible AChE inhibitor used to differentiate between myasthenia gravis, Lambert-Eaton myasthenic syndrome and cholinergic crisis (the so called Tensilon test). Edrophonium, which also possesses a direct nicotinic effect, improves the muscular weakness in patients with myasthenia gravis, whereas it has no effect or worsens weakness in the other conditions.

AChE inhibitors are usually employed in the early stage of myasthenia gravis, when an adequate number of nicotinic receptors still exists [141]. Even though no randomised controlled trial has been conducted on the use of the inhibitors, pyridostigmine seems to be better tolerated and more widely used than neostigmine [142]. Although possessing one quarter of the potency of neostigmine, pyridostigmine is the drug of choice also due to its long duration of action (6 h) and fewer gastrointestinal side effects [143]. Besides, physostigmine is sometimes used in the treatment of primary and secondary glaucoma. It facilitates the drainage of aqueous humour, thus lowering intraocular pressure. Neostigmine is used mainly for reversing neuromuscular blockade, but may be used in other conditions as well such as in colonic pseudo-obstructive conditions [144]. Ambenonium is an interesting compound with the highest affinity to the AChE, despite the fact that it forms only non-covalent bounds [145]. Ambenonium, due to its efficacy, became a parent structure for the derivatives capable to cross the blood brain barrier and which are useful pharmaceuticals in the treatment of AD [146]. Sometimes, the long lasting distigmine**,** which additionally and directly interacts with muscarinic and nicotinic receptors, may also be used; *e.g*. for the treatment of detrusor under-activity [147]. Quaternary derivatives used in the treatment of myasthenia gravis was reviewed by Komloova *et al*. [148].

CONCLUDING REMARKS

Most pharmaceuticals targeting muscarinic receptors are employed at such large doses that no selectivity can be expected. However, some differences in the adverse effect

profile of muscarinic antagonists may still be explained by the variation of expression of muscarinic receptor subtypes in different organs; *e.g*., an antagonist with a muscarinic M3 receptor selective profile affects urinary bladder contraction more than salivation which is exerted by activation of both M1 and M3 receptors. However, a complex pattern of interactions between muscarinic receptor subtypes occurs, as indicated in Fig. **1**, that needs to be considered when searching for selective pharmaceuticals. In the development of new entities for the treatment of for instance pesticide intoxication, the muscarinic receptor selectivity needs to be considered. Reactivators generally have a muscarinic M2 receptor acting profile. Such a blockade may engrave the situation since it may enlarge the effect of the muscarinic M3 receptor effect. This may explain why respiratory arrest is the major cause for deaths by esterase blocking. Thus, in future drug development, muscarinic receptor subtype effects and interactions ought to be considered to a greater degree.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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