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Enhancement of purinergic neurotransmission by galantamine and other acetylcholinesterase inhibitors in the rat vas deferens

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Abstract

Galantamine, a mild acetylcholinesterase inhibitor and an allosteric ligand of nicotinic receptors, enhanced in a concentration-dependent manner the amplitude of purinergic twitch contractions of the electrically stimulated rat vas deferens (0.2 Hz, 1 ms, 60 V). Other acetylcholinesterase inhibitors also increased the twitches, showing a hierarchy of potencies of galantamine>physostigmine >tacrine>rivastigmine=donepezil. The potentiations seem to be unrelated to the ability to inhibit acetylcholinesterase, since the hierarchy of potencies to block the enzyme in vas deferens was tacrine>physostigmine>rivastigmine>donepezil>galantamine. Acetylcholine also increased the twitches; such effect was produced by a low range of concentrations of acetylcholine (10^{-10}–10^{-7} M). This facilitatory effect of acetylcholine on twitches was significantly potentiated by galantamine (10^{-7}–10^{-6} M), but not by rivastigmine or donepezil. A striking enhancement of twitches was also caused by charybdotoxin, a blocker of high-conductance Ca^{2+}-activated K^+ channels, and by 4-aminopyridine, a non-specific blocker of K^+ channels; in addition, apamin, a blocker of small-conductance Ca^{2+}-activated K^+ channels, induced a lower potentiation. The antagonist mecamylamine (10^{-7}–10^{-6} M) reduced by 80% the potentiation by galantamine, indicating the involvement of nicotinic receptors. Therefore, it is suggested that, besides an inhibition of acetylcholinesterase, some additional mechanisms, such as blockade of Ca^{2+}-dependent K^+ channels, or activation of nicotinic receptors of nerve terminals, might be involved in twitch potentiation. These results are relevant in the context of the clinical use of galantamine to improve cognition and behaviour in patients with Alzheimer's disease.

Keywords: Purinergic neurotransmission; Galantamine; Vas deferens

1. Introduction

Acetylcholinesterase inhibitors have been the main therapeutic agents used to treat patients suffering of Alzheimer's type dementia for the last 10 years. Since the pioneering use of tacrine (Summers et al., 1981), other acetylcholinesterase inhibitors such as rivastigmine (Enz et al., 1991), donepezil (Feldman et al., 2001) and galantamine (Coyle and Kershaw, 2001) have sequentially been introduced in the clinic. Their indication emerged from the rationale that Alzheimer's patients have a loss of cholinergic neurons at the basal forebrain and prefrontal cortex, as well as a deep impairment of cholinergic neurotransmission; this might constitute the neurochemical basis for the central symptoms of the disease, such as aphasia, agnosia, apraxia, cognition impairment, behavioural changes and dementia (Perry et al., 1994).

The four clinically useful agents referred to above have in common their ability to inhibit the activity of brain acetylcholinesterase. However, their potencies to block the enzyme sharply differ, donepezil being one of the most potent and galantamine the least (Arnal et al., 1990;

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Rakonczay and Kovacs, 1998; Ogura et al., 2000). On the other hand, substantial differences have been found when comparing the relative potencies of these agents as enzyme antagonists with the potencies in clinical trials to test their benefits on cognition and behaviour in Alzheimer's patients. Thus, the idea is emerging that in addition to inhibiting acetylcholinesterase, these compounds might have other effects. Thus, for rivastigmine its dual action to cause inhibition of acetylcholinesterase and pseudocholinesterase has been proposed to be responsible for its clinical efficacy in Alzheimer's patients (Giacobini et al., 2002). In the case of galantamine, in addition to its mild acetylcholinesterase inhibitory properties, a so-called allosteric potentiating ligand effect has been described (Maelicke et al., 2000). This allosteric modulation is expressed as an enhanced sensitivity of neuronal cholinergic nicotinic receptors that may lead to an augmentation of neurotransmitter release, as it happens to be the case for γ-aminobutyric acid (GABA) and glutamate release in hippocampal slices (Santos et al., 2002). Although this enhanced neurotransmitter release has been attributed to an action on nicotinic receptors (Santos et al., 2002), we have recently found that micromolar concentrations of galantamine also block small-conductance Ca^{2+}-activated K^+ channels of chromaffin cells (Alés et al., 2002), thereby mimicking the effects of apamin (Artalejo et al., 1993).

In the light of the results described for acetylcholinesterase inhibitors, we thought that a comparative study of tacrine, rivastigmine, donepezil, galantamine and physostigmine on a well-defined synapse, such as that of the rat vas deferens, was desirable. The prostatic segment of the vas deferens was used in all experiments of this study. In this segment, the contraction (twitch response) is mostly due to the release of adenosine 5′-triphosphate (ATP) from sympathetic nerve terminals, as a consequence of action potentials elicited by electrical field stimulation (Burnstock, 1986, 1990, 1995). This is so because the twitches are known to be abolished by 1 μM tetrodotoxin, a blocker of Na^+ membrane channels, or by 10 μM suramin, an antagonist of purine P_{2x} receptors (Vladimirova et al., 1994). It is known that in prostatic segment the sensitivity to noradrenaline is substantially lower than that to ATP (Rodhe et al., 1986), indicating that noradrenaline co-released with ATP during each electrical pulse does not significantly contribute to the twitch contractions, as shown by the fact that the α_1 and α_2-adrenoceptors blocker phentolamine does not block such responses (Driessen et al., 1993).

We have recently shown, using the patch-clamp technique, that functional K^+ channels are present in vas deferens (Harhun et al., 2003). Considering that K^+ channel blockers induce effects in vas deferens (Wakade et al., 1975; Docherty and Brady, 1995), and that galantamine blocks K^+ channels of chromaffin cells (Alés et al., 2002; Artalejo et al., 1993), we thought it worthwhile to compare the effect of cholinesterase blockers with that of K^+ channel antagonists.

Cholinergic receptors, either muscarinic (Jurkiewicz et al., 1969, 1976; Souza Brito and Jurkiewicz, 1988; Miranda et al., 1994, 1995) or nicotinic (Carneiro and Markus, 1990), are present in rat vas deferens. Presynaptic nicotinic cholinoceptors (Starke et al., 1991; Todorov et al., 1991) enhance the release of neurotransmitters in several tissues, including the vas deferens. Considering these findings and the fact that therapeutic effects of galantamine in Alzheimer's patients were ascribed to an APL effect on nicotinic receptors (Maelicke et al., 2000), we also decided to study the influence of nicotine on the effects of galantamine and K^+ channel blockers in vas deferens.

The results here obtained indicate that the effects of these acetylcholinesterase blockers in neurotransmission cannot be attributed only to the enzymatic inhibition.

2. Materials and methods

2.1. Rat vas deferens preparation

The experimental procedures used in this work were approved by the Ethics Committee of UNIFESP. Male Wistar rats (280–340 g) were killed by ether inhalation, and the vasa deferentia removed and cleaned of adherent tissues (Jurkiewicz and Jurkiewicz, 1976) and kept in aerated nutrient solution (mM: NaCl 138, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15, and glucose 5.5, in glass-distilled water). The epididymal portion of the vas deferens was discarded, and the prostatic portion was suspended vertically between two parallel platinum electrodes in a 10 ml organ bath, in aerated solution at 30 °C, under a load of usually 1.0 g. The organ was electrically stimulated with pulses of 60 V, 1 ms duration and 0.2 Hz, using a Grass S88 stimulator (Driessen et al., 1993; Vladimirova et al., 1994). The twitches were measured and recorded by means of FT-302 transducers (CB Sciences, USA) coupled to a Power Lab/800 (AD instruments, USA) and a PC computer.

2.2. Contractions induced by electrical field stimulation

We initially selected the parameters of electrical field stimulation that elicited reproducible and stable twitch responses. Thus, when stimulation was made at 0.2 Hz, 1 ms, 60 V, the twitch contractions remained with similar amplitude during at least 2 h of continued recording. In general, 15 min after starting stimulation, when the twitches attained a steady control amplitude, a single or cumulative dose of galantamine was added to the organ bath and maintained until the potentiation attained equilibrium (usually less than 5 min for a single dose), followed by washout of the organ bath. A similar procedure was used when other acetylcholinesterase inhibitors, namely rivastigmine, tacrine, donepezil and physostigmine were used instead of galantamine. In

addition, to investigate the possible action of galantamine on presynaptic K^+ channels, the effects of the small conductance Ca^{2+}-activated K^+ channels blocker apamin, the high-conductance Ca^{2+}-activated K^+ channel blocker charybdotoxin, and the non-specific K^+ channel blocker 4-aminopyridine were used instead of galantamine. Furthermore, to investigate the action of galantamine and other agents on presynaptic nicotinic receptors, the effects of these drugs were also studied on twitch facilitation induced by acetylcholine. In this case, the drugs were added in the presence of a maximum cumulative dose of acetylcholine (usually 10^{-7} M). Finally, the effects of galantamine and K^+ channel blockers were studied on facilitation induced by a single dose of nicotine (usually 10^{-5} M), a classical and selective agonist of nicotinic receptors.

In other experiments with galantamine, the preparation was previously treated with the antagonist of purine P_{2x} receptors suramin (10^{-5} M, 30 min) or of nicotinic receptors mecamylamine (10^{-7}–10^{-6} M, 30 min). In another group, the effects of galantamine were studied on contractions elicited by exogenously administered ATP instead of electrical stimulation.

2.3. Acetylcholinesterase activity

To investigate the inhibitory effects of galantamine upon acetylcholinesterase of vas deferens homogenates, the activity of this enzyme was measured using a photometric method (Ellman et al., 1961). Homogenates were prepared in ultraturrax (3×1 min at 20,000 rpm) using samples of four vasa deferentia (Caricati-Neto et al., 1992; Castillo et al., 1992). Aliquots of 400 μl were incubated in phosphate buffer (pH 8, 37 °C) with acetylcholinesterase substrate acetylthiocholine and dithiobisnitrobenzoate reagent. The acetylcholinesterase activity was measured by following the increase of yellow colour produced from acetylthiocholine when it reacts with dithiobisnitrobenzoate (Ellman et al., 1961). Acetylcholinesterase activity was assayed in the absence or presence of various concentrations of galantamine or other acetylcholinesterase inhibitors.

2.4. Drugs and chemicals

The following drugs were used: galantamine (kindly given by Jansen Pharmaceutica, Beerse (Belgium), riva-

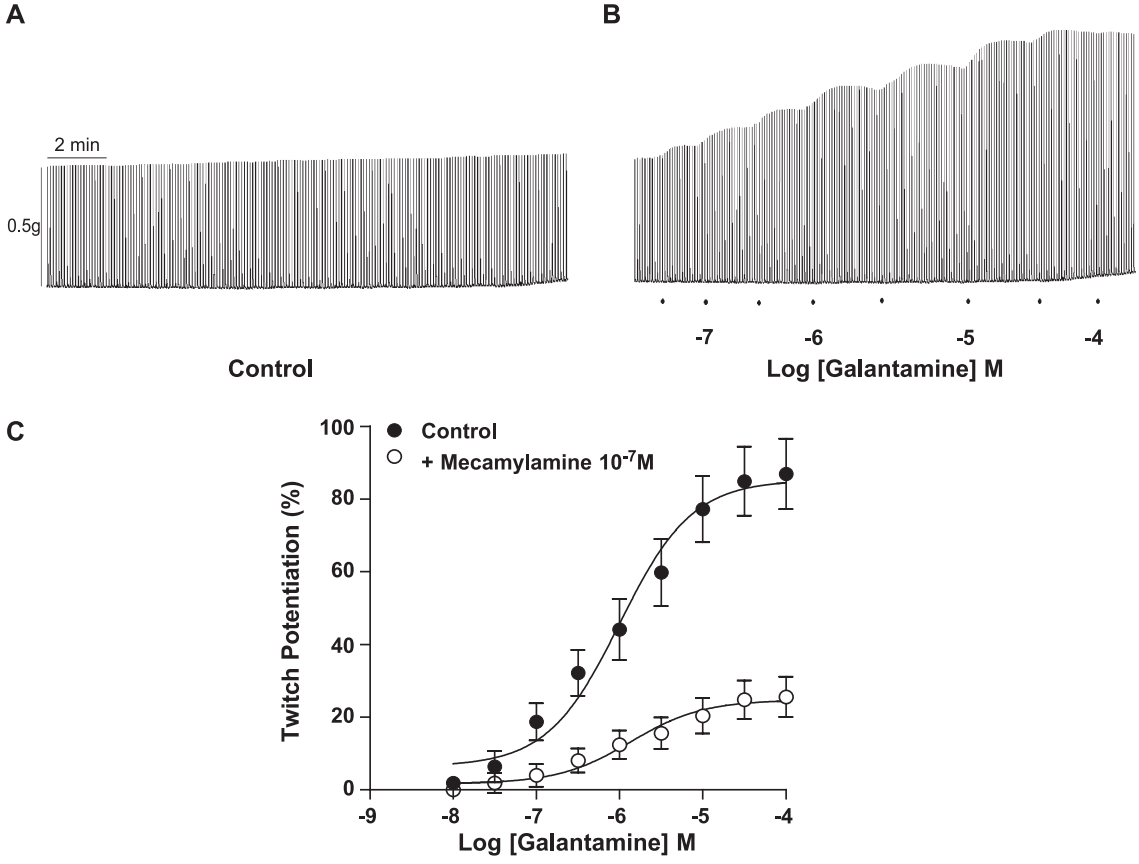

Fig. 1. Enhancement by galantamine of twitch contractions induced in the prostatic segment of the rat vas deferens. (A) Part of original control recording of a preparation continuously stimulated at 0.2 Hz, 1 ms, 60 V, in the absence of galantamine. (B) After an initial 15-min equilibration period, another vas deferens was continuously stimulated while cumulative concentrations of galantamine (3×10^{-8} to 10^{-4} M) were added. (C) Mean concentration–response curve representing the enhancement by galantamine of the amplitude of twitch contractions. This potentiation was calculated as percent increments above the control twitch amplitude before galantamine. Data are means±S.E.M. of six experiments.

stigmine (purified at Instituto Teófilo Hernando, Spain, from Exelon syrup from Novartis Pharmaceutica), tacrine from Aldrich Chem (USA), donepezil, from A&A Pharmachem. (Canada), physostigmine, nicotine, acetylcholine, mecamylamine, adenosine 5′ triphosphate, acetylthiocholine, dithiobisnitrobenzoate, suramin from Sigma (USA), apamin and charybdotoxin, from Research Biochem. International, Natick (USA), and 4-aminopyridine, from Merck (USA). All drugs were dissolved in water.

2.5. Statistics

To quantify the degree of twitch potentiation, the increments of amplitude were measured in each individual experiment. Concentration–response curves were obtained by expressing potentiations induced by each dose as % contractions in relation to the initial twitches obtained in the absence of drugs. Histograms were drawn for maximal potentiations. Student's paired *t*-test and analysis of variance (ANOVA) were performed to determine statistical significance of evaluated parameters. In general, means±SEM were compared, and the significance level was considered as $P<0.05$.

3. Results

3.1. Contractions elicited by electrical field stimulation of the rat vas deferens: potentiation by galantamine

In a large number of vasa deferentia, the mean amplitude of twitch contractions, after a 15-min initial equilibration period (Fig. 1A), was 0.5±0.05 g (n=87). The effects of cumulative concentrations of galantamine on twitch contractions were tested as shown in Fig. 1B. Galantamine enhanced the amplitude of the twitches in a concentration-dependent manner. Some increase of the response was already produced at 3×10^{-8} M and the maximum augmentation occurred at 3×10^{-5} M; larger single or cumulative concentrations of galantamine, up to 10^{-4} M, did not enhance further the twitch amplitude. The resulting mean concentration–response curve is shown in Fig. 1C; from this curve, we calculated the potency (EC_{50}) for galantamine as being 1.0±0.17 μM.

It is noteworthy that galantamine did not induce contractions of the vas deferens, if given in the absence of electrical stimulation. In addition, in the presence of electrical stimulation, the baseline contraction was not elevated by galantamine, in spite of the fact that it practically doubled the amplitude of twitches (Fig. 1B). These results indicate that the effect of galantamine is not postsynaptic, since in this case we would expect a rise of the baseline (Rodhe et al., 1986), either in absence or presence of twitch contractions.

In another group of experiments, contractions produced by addition of ATP (10^{-4} M) to the organ bath, in the absence of twitches, attained a value of 0.36±0.06 g (n=4) and were not potentiated by galantamine 10^{-5} M (0.31±0.04 g, n=4), indicating that the potentiation of twitches previously described for galantamine was not due to potentiation of released ATP.

The nicotinic receptor antagonist mecamylamine (10^{-7}–10^{-6} M) drastically reduced the potentiation by galantamine by about 80% (Fig. 1C), indicating that these effects involved nicotinic receptors. However, in absence of galantamine, the amplitude of twitches was not modified by mecamylamine

Table 1
Summary of the effects of acetylcholinesterase (AChE) inhibitors described so far in the literature and in this study

Drugs	AchE inhibition (IC_{50}, nM)		Potentiation of purinergic twitches (% E_{max})*	Other effects
	Brain	Vas deferens*		
Tacrine	77[a], 80[b] 93[c], 450[d]	100±20 (n=3)	43±06	–
Physostigmine	0.7[a], 18[d] 60[b], 251[c]	700±80 (n=3)	68±11	–
Rivastigmine	–	2100±220 (n=3)	7±5	Blockade of BuChE[e]
Donepezil	7[a], 13[c]	4300±510 (n=3)	4±1	–
Galantamine	1995[c]	15,800±3270 (n=3)	89±12	Blockade of K^+ channels[f]

BuChE—Butirylcholinesterase.
[a] Rat brain (Ogura et al., 2000).
[b] Mouse brain (Arnal et al., 1990).
[c] Rat cortex (Tang, 1996).
[d] Human cortex (Rakonczay and Kovacs, 1998).
[e] Human cerebrospinal fluid (Giacobini et al., 2002).
[f] Chromafin cells (Alés et al., 2002).
* Data obtained in the present work.

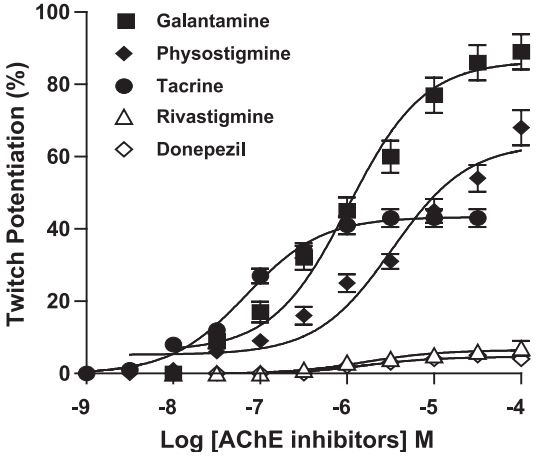

Fig. 2. Comparative effects of various acetylcholinesterase inhibitors on the twitch contractions evoked by electrical field stimulation (0.2 Hz, 1 ms, 60 V) in the rat vas deferens, as shown in the previous figure. The experiments were performed following the protocols used for galantamine in Fig. 1, using the range of concentrations for each of the five compounds indicated in the figure. The ordinate represents the increase of the basal twitch contraction elicited by each compound, expressed as % above control amplitude. Data are means±S.E.M. of at least six experiments.

(10^{-7} M), being similar in its absence (0.38±0.05 g, n=4) or presence (0.35±0.03 g, n=4), showing that this nicotinic blocker isolatedly does not influence the purinergic neurotransmission. Finally, the purine P_{2x} receptor antagonist suramin (10^{-5} M) completely blocked both the twitches and its enhancement by galantamine (not shown). This shows that ATP released from nerve terminals is the only neurotransmitter responsible for the generation of twitch contractions, both in the absence and presence of galantamine, by acting on purine P_{2x} receptors.

3.2. Comparative effects on twitch contractions of galantamine, rivastigmine, tacrine, donepezil and physostigmine

The enhancement by galantamine of the amplitude of contractions was compared with those of the other four acetylcholinesterase inhibitors tested in this study, using protocols similar to that shown in Fig. 1. The corresponding concentration–response curves are shown in Fig. 2, as a function of the ability of each compound to enhance the control twitch response. The maximum enhancement of the twitch responses by these compounds considerably varied (Table 1). For instance, the maximal dose of tacrine and physostigmine enhanced the response by about 43% and 68%, respectively. On the other hand, donepezil and rivastigmine caused an almost negligible potentiation (lower than 7%). In the latter experiments, the responses were significantly increased when galantamine (10^{-5} M) was added in the presence of maximal doses of donepezil or rivastigmine (not shown), indicating that the lack of potentiation was not due to technical failure, nor to tissue damage.

3.3. Enhancement of the twitch responses elicited by galantamine and K^+ channel blockers, in absence and presence of nicotine

Fig. 3A–C shows original records taken from experiments aimed at testing the effects of single concentrations of

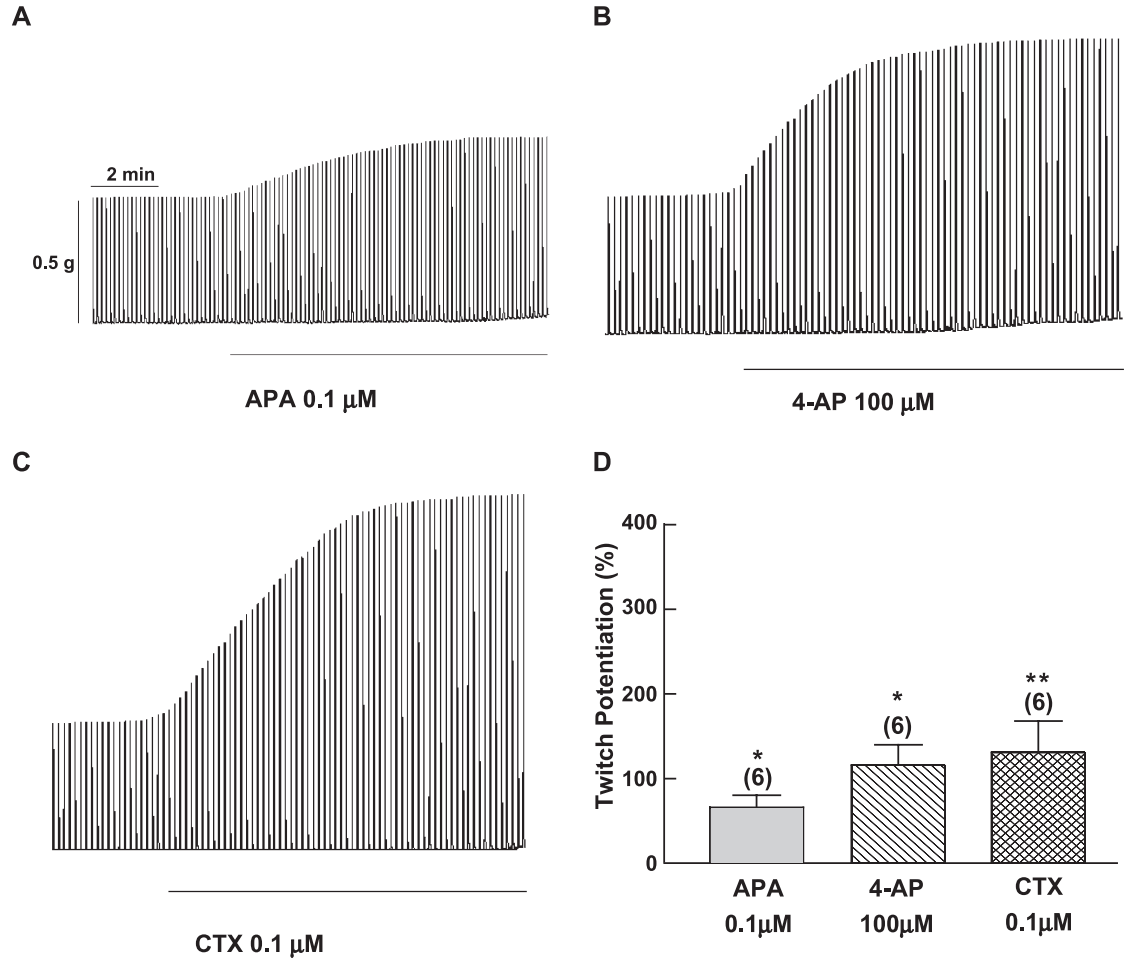

Fig. 3. Enhancement by K^+ channel blockers of twitch contractions induced by electrical field stimulation (0.2 Hz, 1 ms, 60 V) in the prostatic segment of the rat vas deferens. Panels A, B and C show original records of the effects of apamin (APA), 4-aminopyridine (4-AP), and charybdotoxin (CTX), respectively. Horizontal lines indicate the presence of the blocker. Panel D shows the corresponding potentiation (means±S.E.M) elicited by each compound. The y-axis represents the potentiation as a percent increase of twitch amplitude in relation to the twitch in absence of the blocker. Number of preparations shown in parenthesis. *P<0.05, **P<0.01 compared with control without drugs.

the K^+ channel blockers apamin, 4-aminopyridine and charybdotoxin. The corresponding twitch potentiations induced by these drugs, expressed as percentages, are represented in Fig. 3D. In this case, the largest potentiation was induced by the block of high-conductance Ca^{2+}-activated K^+ channels by charybdotoxin.

Fig. 4A–D shows that nicotine (10^{-5} M) rapidly enhanced the amplitude of twitch responses by about 50%. A significant additional increment of the twitches was induced by 4-aminopyridine and charybdotoxin, when incubated after the maximal effect of nicotine (Fig. 4B and C). However, galantamine (10^{-6} M) and apamin (10^{-7} M)

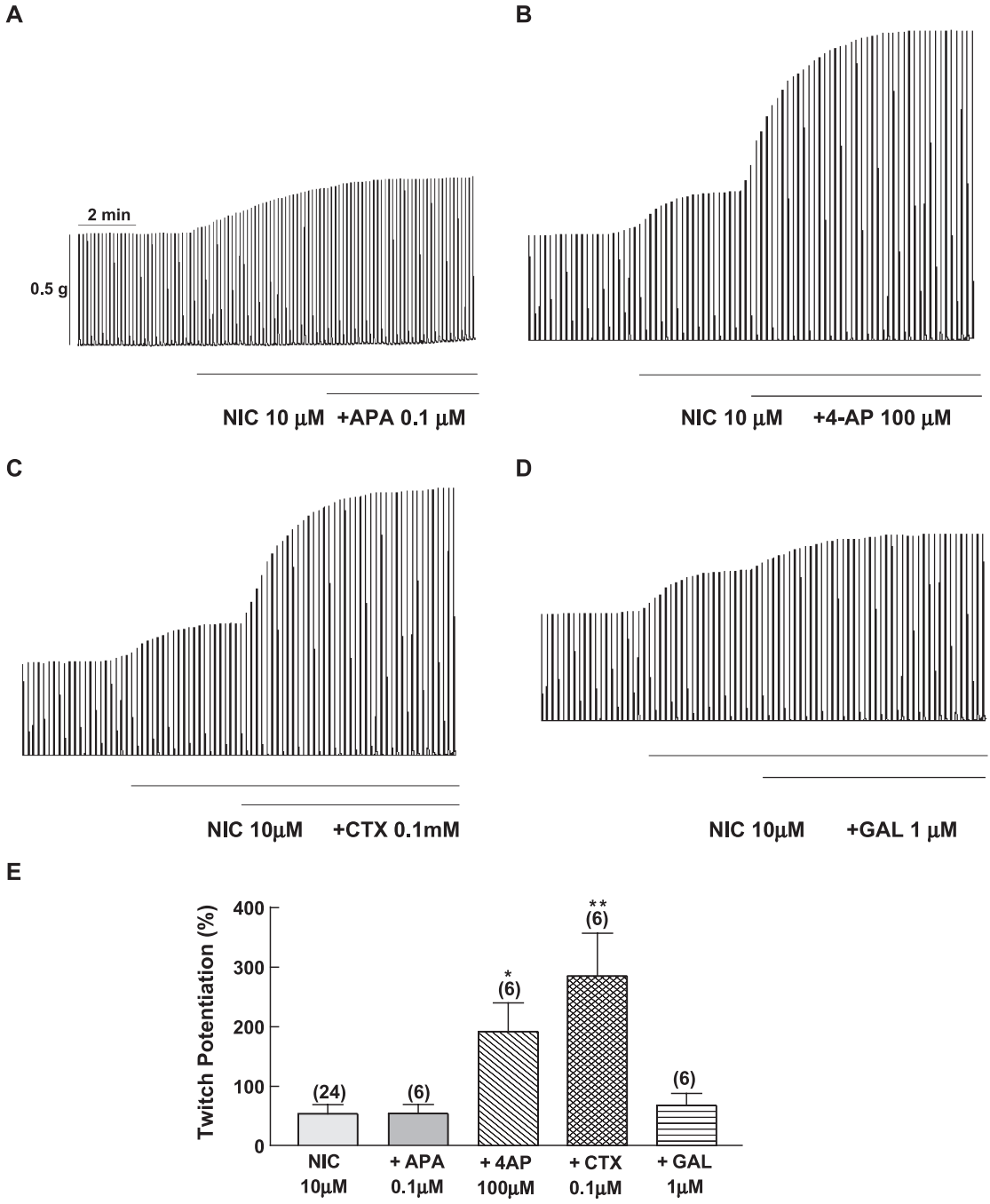

Fig. 4. Enhancement induced by nicotine, galantamine and K^+ channel blockers of the twitch contractions evoked by electrical field stimulation (0.2 Hz, 1 ms, 60 V) in the prostatic segment of the rat vas deferens. Panels A, B, C and D show original records of the effects of apamin (APA), 4-aminopyridine (4-AP), charybdotoxin (CTX) and galantamine (GAL) on the maximal potentiation of twitches induced by nicotine (NIC), respectively. Upper horizontal lines indicate the presence of nicotine, and the lower lines of the other agents, respectively. Panel E shows the potentiation (mean±S.E.M.) elicited by each compound in the presence of the potentiation induced by NIC. The *y*-axis represents the potentiation as a percent increase of twitch amplitude in relation to the twitch in absence of nicotine. Number of preparations shown in parentheses. **P*<0.05, ***P*<0.01 compared to NIC alone.

induced negligible effects when added after the maximal effect of nicotine (Fig. 4A and D). The increment of the twitches induced by apamin, 4-aminopyridine, charybdotoxin and galantamine on the potentiation by nicotine, is represented in Fig. 4E. In summary, neither apamin nor galantamine enhanced significantly the twitch response above the level achieved by nicotine; in contrast, 4-aminopyridine and charybdotoxin caused two- to threefold increases of the twitch responses.

3.4. Effects of acetylcholinesterase inhibitors on the potentiation of twitch responses by acetylcholine

Similarly to nicotine, acetylcholine enhanced the amplitude of twitch responses induced by electrical stimulation. In these experiments, we wished to correlate the effects of acetylcholinesterase inhibitors on acetylcholine potentiation and on the acetylcholinesterase activity of rat vas deferens homogenates (see Section 3.5, below). In addition, we wished to see the maximum acetylcholine twitch potentiation at the lowest possible acetylcholine concentrations. Hence, a submaximal voltage stimulation was used to elicit twitch contractions, i.e., 40 mV instead of the 60 mV of previous experiments. Under these conditions, an important twitch potentiation was observed, already at subnanomolar concentrations of acetylcholine. These concentrations are relevant from the physiological point of view but also to correlate the functional effects of the inhibitors (i.e., enhancement of acetylcholine potentiation) with their potency to inhibit acetylcholinesterase activity.

Fig. 5A shows that acetylcholine enhanced the twitch responses. The respective concentration–response curve for the potentiation by acetylcholine shows that the threshold concentration was as low as 10^{-10} M, and the maximal

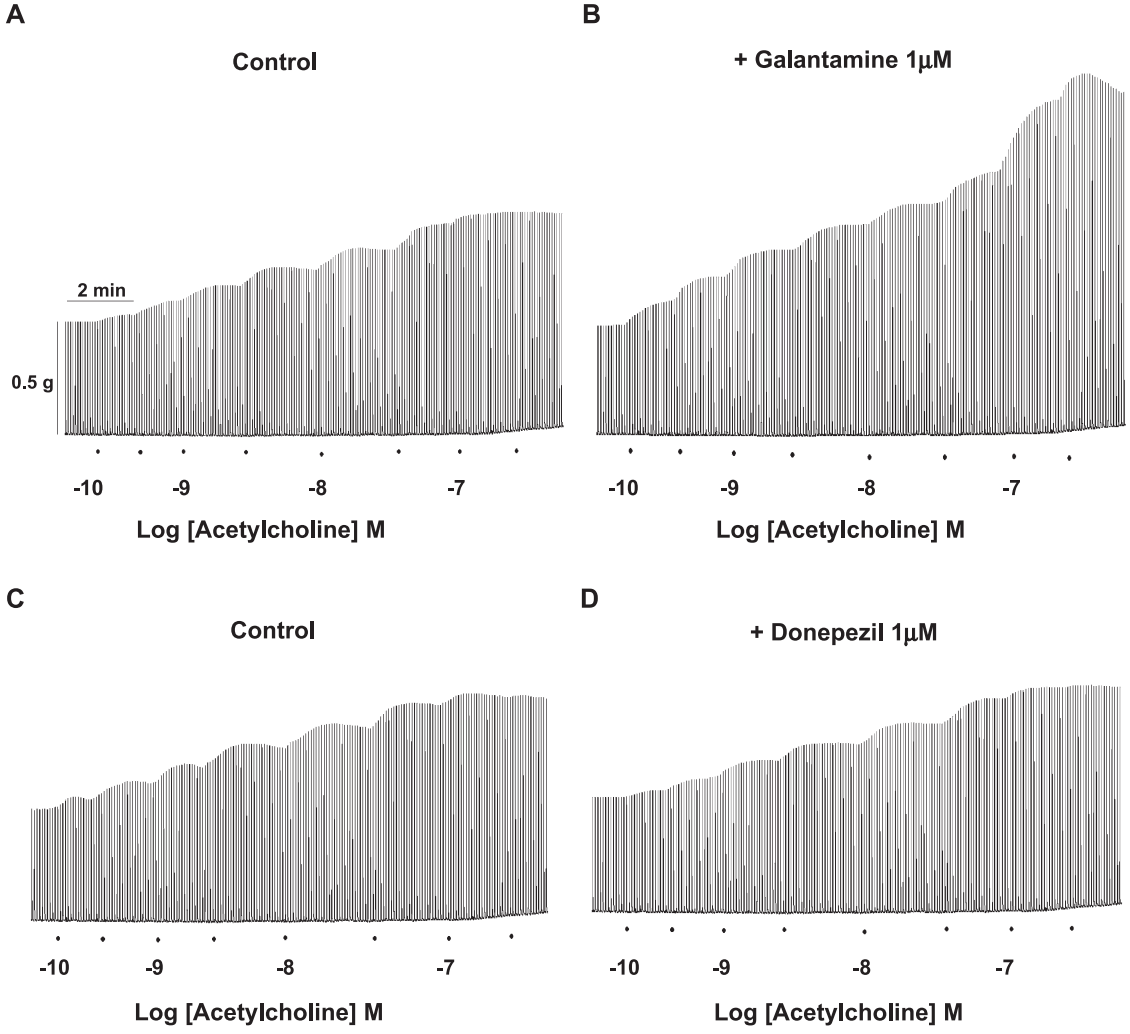

Fig. 5. Effects of galantamine and donepezil on the facilitation by acetylcholine of the twitch contractions evoked by electrical stimulation (0.2 Hz, 1 ms, 40 V) in the prostatic segment of the rat vas deferens. (A) Original record showing the augmentation of the amplitude of twitch responses, evoked by cumulative increasing concentrations of acetylcholine, added as indicated by the dots at the bottom. (B) Same preparation as A, in which the concentration–response curve to acetylcholine was performed in the presence of galantamine, added 5 min before and during the addition of acetylcholine. C and D show an experiment similar to that in A and B, except that donepezil was used instead of galantamine.

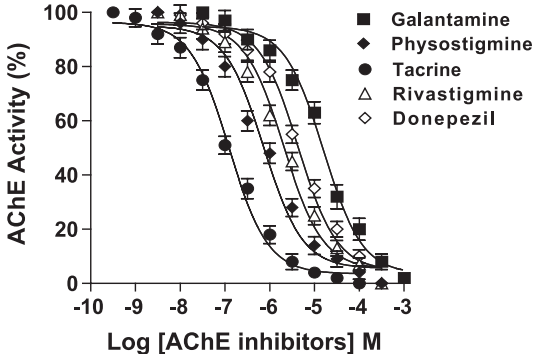

Fig. 6. Effects of acetylcholinesterase (AChE) inhibitors on the acetylcholinesterase activity of rat vas deferens homogenates. Acetylcholinesterase activity and its inhibition by increasing concentrations of each compound were determined as described in Materials and methods (see Table 1 for the IC_{50} of each compound tested). Data are means±S.E.M. of three different experiments performed in quadruplicate.

potentiation was 10^{-7} M. Concentrations of 10^{-7} M or higher started to increase the basal tonus of the preparation, indicating the involvement of a postsynaptic component at this point. Fig. 5B shows that galantamine (10^{-6} M), preincubated for 5 min, potentiated the effects of acetylcholine. In this case, the effects produced by subnanomolar acetylcholine concentrations were significantly increased, as well. The same protocol was used to study donepezil effects on twitch potentiation induced by acetylcholine (Fig. 5C and D). Fig. 5D shows that 10^{-6} M donepezil produced only a negligible effect on the acetylcholine potentiation of twitch responses. Other concentrations of donepezil (10^{-7} to 10^{-5} M) were equally ineffective in the same preparation; these concentrations were added in a cumulative mode, and concentration–response curves of acetylcholine tested in its presence (data not shown).

3.5. Effects of acetylcholinesterase inhibitors on enzymatic activity in vas deferens homogenates

Concentration–inhibition curves of acetylcholinesterase were obtained by using the acetylcholinesterase inhibitors in homogenates of rat vas deferens, as shown in Fig. 6. Note that tacrine was the most potent inhibitor while galantamine was the least potent. The values of inhibitory potency (IC_{50}) for the five compounds tested are shown in Table 1. In this case tacrine was followed by physostigmine, rivastigmine, donepezil and galantamine, respectively.

4. Discussion

The present work demonstrates that electrically evoked contractions of rat vas deferens were increased by (a) acetylcholinesterase inhibitors, (b) agonists of nicotinic receptors, and (c) blockers of high-conductance Ca^{2+}-activated K^+ channel. The inhibitors of acetylcholinesterase have in common their ability to inhibit this enzyme activity in various mammalian brain preparations (see Table 1 for references) and in rat vas deferens homogenates (this study). In view of these well-recognized effects, one is tempted to ascribe the potentiating action of these agents, on the twitches of the rat vas deferens, to their ability to inhibit the acetylcholinesterase activity of this organ.

Because the rat vas deferens is an excellent model for the study of the noradrenergic and purinergic components of sympathetic co-transmission (Burnstock, 1986, 1990, 1995), we decided to study the effects of galantamine on purinergic transmission in this preparation. In rodent vas deferens, purinergic contractions (twitches) are induced by stimulation of postsynaptic purine P_{2x} receptors by ATP released by electrical field stimulation of sympathetic nerves (Burnstock, 1995). In spite of the predominant role of purinergic transmission during electrical nerve stimulation of the vas deferens, endogenous acetylcholine could also be released during each twitch; in fact, the presence of cholinergic nerves in this preparation is well documented (Robinson, 1969; Gosling and Dixon, 1972; Sjostrand, 1981). Hence, even minute amounts of endogenously released acetylcholine could be contributing to the regulation of the control amplitude of the twitches, by acting on presynaptic nicotinic receptors; this is supported by our observation that the exogenous application of acetylcholine at subnanomolar concentrations already enhanced the twitch amplitude at concentrations of 10^{-10} M. Hence, it is expected that an acetylcholinesterase inhibitor, by delaying the hydrolysis of acetylcholine, will enhance its twitch potentiating effect. While this prediction seems to be correct, the various acetylcholinesterase inhibitors used here enhanced, however, to different extents, the effects of acetylcholine. These differences indicate that the different potentiations are not simply due to the different potencies to inhibit the vas deferens acetylcholinesterase; thus, in spite of being the least potent acetylcholinesterase inhibitor, galantamine was the most potent enhancer of twitch contractions (see Fig. 2 and Table 1) and of the acetylcholine-evoked twitch potentiation. In contrast, rivastigmine and donepezil, which were 10-fold more potent than galantamine in inhibiting acetylcholinesterase, little enhanced the twitch responses of the twitch-potentiating effects of acetylcholinesterase.

A second possibility to explain the facilitation of twitch responses by galantamine is its ability to block K^+ channels (Sah, 1996). In rat chromaffin cells galantamine exhibits an apamin-like effect, enhancing the acetylcholine- and the K^+-evoked catecholamine release and increasing the intracellular concentration of free Ca^{2+} ions (Alés et al., 2002). We have shown that more than one type of functional K^+ channels is present in vas deferens (Harhun et al., 2003). In the present study, we observed that apamin, at the concentration of 0.1 μM that fully blocks the currents mediated by small conductance Ca^{2+}-activated K^+ channel in chromaffin cells (Artalejo et al., 1993), enhanced the

twitch responses by about 40%; in fact, galantamine augmented such responses when added on top of apamin, suggesting that the effect of galantamine was unrelated to the blockade of small conductance Ca^{2+}-activated K^+ channel. Charybdotoxin, a selective blocker of high-conductance Ca^{2+}-activated K^+ channels (Sah, 1996), clearly potentiated the twitches. The corresponding concentration–response curves were similar to that for galantamine, although charybdotoxin was about 10-fold more potent. However, competition experiments with both compounds showed that charybdotoxin still potentiated the twitch responses when added on top of galantamine; this was not true when the compounds were added the opposite way, first charybdotoxin and then galantamine (not shown). Thus, it is still uncertain whether galantamine has a charybdotoxin like effect in potentiating the twitches; however, since charybdotoxin-sensitive K^+ currents are present in isolated smooth muscle cells of this preparation (Harhun et al., 2003), further experiments might tell us whether or not galantamine is blocking these currents.

The third possibility to explain the mechanism of action of galantamine in enhancing the twitch responses relies on presynaptic nicotinic receptors. Several studies showed that agonists of nicotinic receptors, such as nicotine and carbachol, enhance twitch responses in the rat vas deferens and that such effect was blocked by nicotinic antagonists (Carneiro and Markus, 1990; Todorov et al., 1991). These findings agree with the idea that presynaptic nicotinic receptors augment the release of neurotransmitters from sympathetic nerve terminals (Kirpekar et al., 1980). In this study, we have corroborated that the nicotinic agonist nicotine and acetylcholine enhance the twitch responses and that this effect was antagonized by the nicotinic blocker mecamylamine. What seemed surprising in our present results is that minute concentrations of nicotine (1 μM) but particularly of acetylcholine (0.1–1 μM) already enhanced the twitch responses. This is rather relevant, since nicotinic receptors are known to desensitize quickly when interacting with the respective agonists, in a concentration- and time-dependent manner. Thus, it is unlikely that such a small concentration of acetylcholine, as 1 nM, is causing desensitization of nicotinic receptor even after long-term exposures. In fact, after this dose of acetylcholine, the small potentiation (20% increase) of twitch responses was maintained for several minutes; this also occurred in the presence of 10^{-7} M galantamine and 10^{-9} M acetylcholine.

Maelicke et al. (2000) coined the expression "allosteric potentiating ligand" to mean an allosteric "sensitization" of nicotinic receptors to acetylcholine, caused by galantamine, physostigmine and some other compounds such as codeine. The results here obtained in the vas deferens fit well within this concept: at submicromolar concentrations that do not block acetylcholinesterase, galantamine enhanced the potentiating effects of nanomolar concentrations of acetylcholine. These low concentrations are relevant because the allosteric "sensitization" of nicotinic receptors seems to occur in a narrow range of rather low concentrations of galantamine (Maelicke et al., 2000). Furthermore, this effect is best seen when also using low concentrations of nicotinic agonists that prevent receptor desensitization, as it happens to be the case in our present experiments.

As stated in Introduction, the twitch contractions of the rat vas deferens are mainly produced by ATP release; thus, their amplitude might be taken as an index of the amount of neurotransmitter released during each stimulus (Todorov et al., 1991). The fact that nanomolar concentrations of acetylcholine enhanced neurotransmission suggests that this may be physiologically relevant to the general concept of cholinergic modulation of neurotransmitter release.

In this context, our experiments are also relevant to explain the therapeutic effects of galantamine in improving behaviour and cognition, in patients suffering of Alzheimer's disease. The brain of these patients undergoes a pronounced loss of cholinergic neurons added to concomitant low levels of acetylcholine (Perry et al., 1994). Thus, our observation that galantamine improves the facilitation of neurotransmission caused by nanomolar concentrations of acetylcholine, is most relevant in the clinical and therapeutic context of Alzheimer's disease. This direct effect of galantamine on nicotinic receptors is likely more relevant than the mere increase of the synaptic concentration of acetylcholine by inhibition of acetylcholinesterase; furthermore, the continued elevation of synaptic acetylcholine concentration would possibly lead to nicotinic receptor desensitization (Maelicke et al., 2000).

Previous studies performed by Mutafova-Yambolieva et al. (1993) have shown that galantamine (0.1–300 μM) increased the spontaneous mechanical activity and exerted by itself an enhancement of smooth muscle tone in the rat jejunum and ileum, suggesting that this facilitatory effect of galantamine is mediated not only by presynaptic but also by postsynaptic mechanisms. In the present work, the participation of postsynaptic mechanisms on facilitatory effect of galantamine on purinergic neurotransmission could not be detected, because this agent was unable to produce contractions per se or to modify contractile responses induced by exogenous ATP. These results support the view that facilitatory effects of galantamine on purinergic neurotransmission of rat vas deferens are mainly mediated by presynaptic mechamisms.

In conclusion, although it may not be possible to exclude an action on the acetylcholinesterase of vas deferens, the enhancement of purinergic neurotransmission by galantamine might alternatively be due to two other mechanisms, as the allosteric "sensitization" of presynaptic nicotinic receptors or the block of K^+ channels of sympathetic neurons. The later two actions are unique to galantamine and possibly unrelated to its acetylcholinesterase inhibitory properties, that it shares with other drugs used to treat patients with Alzheimer's disease, such as rivastigmine and donepezil.

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References

Alés, E., Arias, E., Garcia, A.G., Lopez, M.G., 2002. Galantamine induces neurotransmitter release likely by blocking SK channels: a new mechanism of action for anti-Alzheimer's drugs. Methods Find. Exp. Clin. Pharmacol. 24, 151–162.

Arnal, F., Cote, L.J., Ginsburg, S., Lawrence, G.D., Naini, A., Sano, M., 1990. Studies on new, centrally active and reversible acetylcholinesterase inhibitors. Neurochem. Res. 15, 587–591.

Artalejo, A.R., Garcia, A.G., Neher, E., 1993. Small conductance Ca^{2+}-activated K^+ channels in bovine chromaffin cells. Pflugers Arch. Eur. J. Physiol. 423, 97–103.

Burnstock, G., 1986. The changing face of autonomic neurotransmission. Acta Physiol. Scand. 126, 67–91.

Burnstock, G., 1990. The fifth Heymans memorial lecture co-transmission. Arch. Int. Pharmacodyn. 304, 7–33.

Burnstock, G., 1995. Noradrenaline and ATP: cotransmitters and neuromodulators. J. Physiol. Pharmacol. 46, 365–384.

Caricati-Neto, A., Sette, M., Jurkiewicz, A., 1992. Increased density of α-adrenoceptors in vas deferens of spontaneously hypertensive rats (SHR), indicated by functional and receptor binding studies. Eur. J. Pharmacol. 218, 51–58.

Carneiro, R.C., Markus, R.P., 1990. Prejunctional nicotinic receptors involved in release of noradrenaline and ATP from the prostatic portion of the rat vas deferens. J. Pharmacol. Exp. Ther. 255, 95–100.

Castillo, C.F.J., Lafayette, S., Caricati-Neto, A., Sette, M., Jurkiewicz, N.H., Garcia, A.G., Jurkiewicz, A., 1992. Low dihydropyridine receptor density in vasa deferentia of castrated rats. Br. J. Pharmacol. 105, 257–258.

Coyle, J., Kershaw, P., 2001. Galantamine, a cholinesterase inhibitor that allosterically modulates nicotinic receptors: effects on the course of Alzheimer's disease. Biol. Psychiatry 49, 289–299.

Docherty, J.R., Brady, G., 1995. Prejunctional actions of K^+ channels blockers in rat vas deferens. Eur. J. Pharmacol. 287, 287–293.

Driessen, B., Von Kugelgen, I., Starke, K., 1993. Neural ATP release and its α_2-adrenoceptor-mediated modulation in guinea-pig vas deferens. Naunyn-Schmiedeberg's Arch. Pharmacol. 348, 358–366.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Enz, A., Boddeke, H., Gray, J., Spiegel, R., 1991. Pharmacologic and clinicopharmacologic properties of SDZ ENA 713, a centrally selective acetylcholinesterase inhibitor. Ann. N.Y. Acad. Sci. 640, 272–275.

Feldman, H., Gauthier, S., Hecker, J., Vellas, B., Subbiah, P., Wholen, E., 2001. A 24-week randomized, double blind study of donepezil in moderate to severe Alzheimer's disease. Neurology 57, 613–620.

Giacobini, E., Spiegel, R., Enz, A., Veroff, A.E., Cutler, N.R., 2002. Inhibition of acetyl- and butyryl-cholinesterase in the cerebrospinal fluid of patients with Alzheimer's disease by rivastigmine: correlation with cognitive benefit. J. Neural Transm. 109, 1053–1065.

Gosling, J.A., Dixon, J.S., 1972. Differences in the manner of autonomic innervation of the muscle layers of the guinea-pig ductus deferens. J. Anat. 112, 81–91.

Harhun, M.I., Jurkiewicz, A., Jurkiewicz, N.H., Krysthtal, D.O., Shuba, M.F., Vladimirova, I.A., 2003. Voltage-gated potassium currents in rat vas deferens smooth muscle cells. Eur. J. Phys. 446, 380–386.

Jurkiewicz, A., Jurkiewicz, N.H., 1976. Dual effect of alpha-adrenoceptor antagonists in rat isolated vas deferens. Br. J. Pharmacol. 56, 169–172.

Jurkiewicz, A., Jurkiewicz, N.H., Barros, G.G., Valle, J.R., 1969. Relative responsiveness (ρ): of pharmacological receptor systems in the rat vas deferens. Pharmacology 2, 89–99.

Jurkiewicz, A., Abdo, A.O., Jurkiewicz, N.H., Guedes, A.O., Souccar, C., 1976. Relative responsiveness (ρ): a critical analysis of a new method in receptor differentiation. Gen. Pharmacol. 7, 93–101.

Kirpekar, S.M., Garcia, A.G., Prat, J.C., 1980. Action of nicotine on sympathetic nerve terminals. J. Pharmacol. Exp. Ther. 213, 133–138.

Maelicke, A., Schrattenholz, A., Samochocki, M., Radina, M., Albuquerque, E.X., 2000. Allosterically potentiating ligands of nicotinic receptors a treatment strategy for Alzheimer's disease. Behav. Brain Res. 113, 199–206.

Miranda, H.F., Duran, E., Bustamante, D., Paeile, C., Pinardi, G., 1994. Pre- and postjunctional muscarinic receptor subtypes in the vas deferens of rat. Gen. Pharmacol. 25, 1643–1647.

Miranda, H.F., Duran, E., Fernandez, E., Pinardi, G., 1995. Muscarinic receptor subtypes in the bisected vas deferens of the rat. Gen. Pharmacol. 26, 387–391.

Mutafova-Yambolieva, V.M., Yamboliev, I.V., Mihailova, D.N., 1993. Comparative effects of the anticholinesterase drug galantamine on the mechanical activity of isolated rat jejunum and ileum. Gen. Pharmacol. 24, 1253–1256.

Ogura, H., Kosaka, T., Kuriya, Y., Yamanishi, Y., 2000. Comparison of inhibitory activities of donepezil and other cholinesterase inhibitors on acetylcholinesterase and butyrilcholonesterase in vitro. Methods Find. Exp. Clin. Pharmacol. 22, 609–613.

Perry, E.K., Haroutunian, V., Davis, K.L., Levy, R., Lantos, P., Eagger, S., Honavar, R.M., Dean, A., Griffiths, M., McKeith, J.G., 1994. Neocortical cholinergic activities differentiate Lewy body dementia from classical Alzheimer's disease. NeuroReport 5, 747–749.

Rakonczay, Z., Kovacs, J., 1998. Cholinesterases in Alzheimer's disease and cholinesterase inhibitors in Alzheimer's therapy. Acta Biol. Hung. 49, 55–70.

Robinson, P.M., 1969. A cholinergic component in the innervation of the longitudinal smooth muscle of the guinea-pig vas deferens: the fine structural localization of acetylcholinesterase. J. Cell Biol. 41, 462–476.

Rodhe, G.C., Venezian, E., Huidobro-Toro, J.P., 1986. Assimetric distribution of purinergic and adrenergic neurotransmission cooperates in the motor activity along the rat vas deferens. Neurosci. Lett. 71, 197–202.

Sah, P., 1996. Ca^{++}-activated K^+ currents in neurones: types, physiological roles and modulation. TINS 19, 150–154.

Santos, M.D., Alkondon, M., Pereira, E.F., Aracava, Y., Eisenberg, H.M., Maelicke, A., Albuquerque, E.X., 2002. The nicotinic allosteric potentiating ligand galantamine facilitates synaptic transmission in the mammalian central nervous system. Mol. Pharmacol. 61, 1222–1234.

Sjostrand, N.O., 1981. Smooth muscle of vas deferens and other organs in the male reproductive tract. In: Bulbring, E., Branding, A., Jones, A., Tomita, T. (Eds.), Smooth Muscle. Academic Press, New York, pp. 367–376.

Souza Brito, A.R.M., Jurkiewicz, A., 1988. Some pharmacological properties of the circular smooth muscle layer of the rat vas deferens. J. Pharm. Pharmacol. 40, 781–786.

Starke, K., Bultmann, R., Bulloch, J.R., 1991. Noradrenaline-ATP corelease and cotransmission following activation of nicotinic receptors at postganglionic sympathetic axons. J. Neural Transm. Suppl. 34, 93–98.

Summers, W.K., Viesselman, J.O., Marsh, G.M., Candelora, K., 1981. Use of THA in treatment of Alzheimer's like dementia: pilot study in twelve patients. Biol. Psychiatry 16, 145–153.

Tang, X.C., 1996. Huperzine A (shuangyiping): a promising drug for Alzheimer's disease. Zhongguo Yao Xuebao 17, 481–484.

Todorov, l., Windisch, K., Shersen, H., Lajtha, A., Papasova, M., Vizi, E.S., 1991. Prejunctional nicotinic receptors involved in facilitation of stimulation-evoked noradrenaline release from the vas deferens of the guinea-pig. Br. J. Pharmacol. 102, 186–190.

Vladimirova, I., Jurkiewicz, N.H., Jurkiewicz, A., 1994. Evidence for participation of nitric oxide in excitatory neurotransmitter in rat vas deferens. Life Sci. 55, 1123–1128.

Wakade, A.R., Garcia, A.G., Kirpekar, S.M., 1975. Effect of castration on the smooth muscle cells of the internal sex organs of the rat: influence of the smooth muscle on the sympathetic neurons innervating the vas deferens, seminal vesicle and coagulating gland. J. Pharmacol. Exp. Ther. 193, 424–434.