Experimental Physiology – *Research Paper*

Non-quantal release of acetylcholine from parasympathetic nerve terminals in the right atrium of rats

Denis V. Abramochkin[1,2], Leniz F. Nurullin[3], Anastasia A. Borodinova[1], Nadezhda V. Tarasova[1], Galina S. Sukhova[1], Eugen E. Nikolsky[3,4] and Leonid V. Rosenshtraukh[2]

[1]*Department of Human and Animal Physiology, Moscow State University, Leninskiye Gory, 1, 12, Moscow, Russia*
[2]*Laboratory of Cardiac Electrophysiology, Institute of Experimental Cardiology, Rosmedtechnologiya, 3-rd Cherepkovskaya, 15A, Moscow, Russia*
[3]*Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, Russia*
[4]*Kazan State Medical University, Kazan, Russia*

Acetylcholinesterase (AChE) inhibitors provoke typical cholinergic effects in the isolated right atrium of the rat due to the accumulation of acetylcholine (ACh). Our study was designed to show that in the absence of vagal impulse activity, ACh is released from the parasympathetic nerve fibres by means of non-quantal secretion. The conventional microelectrode technique was used to study changes in action potential (AP) configuration in the right atrium preparation of rats during application of AChE inhibitors. Staining with the lipophilic fluorescent dye FM1-43 was used to demonstrate the presence of endocytosis in cholinergic endings. The AChE inhibitors armin (10^{-7}–10^{-5} M) and neostigmine (10^{-7} to 5×10^{-6} M) caused a reduction of AP duration and prolonged the cycle length. These effects were abolished by atropine and were therefore mediated by ACh accumulated in the myocardium during AChE inhibition. Putative block of impulse activity of the postganglionic neurons by tetrodotoxin (5×10^{-7} M) and blockade of ganglionic transmission by hexomethonium (2×10^{-4} M), as well as blockade of all forms of quantal release with *Clostridium botulinum* type A toxin (50 U ml^{-1}), did not alter the effects of armin. Experiments with FM1-43 dye confirmed the effective block of exocytosis by botulinum toxin. Selective inhibition of the choline uptake system using hemicholinium III (10^{-5} M), which blocks non-quantal release at the neuromuscular junction, suppressed the effects of AChE inhibitors. Thus, accumulation of ACh is likely to be caused by non-quantal release from cholinergic terminals. We propose that non-quantal release of ACh, shown previously at the neuromuscular junction, is present in cholinergic postganglionic fibres of the rat heart in addition to quantal release.

(Received 20 August 2009; accepted after revision 15 September 2009; first published online 18 September 2009)
Corresponding author D. V. Abramochkin: Department of Human and Animal Physiology, Biological Faculty of the Moscow State University, Leninskije Gory, 1, 12, Moscow, Russia. Email: abram340@mail.ru

It is well known that parasympathetic regulation is extremely important for normal functioning of the mammalian heart. Acetylcholine (ACh) is the main neuromediator of postganglionic parasympathetic intracardiac neurons. Acetylcholine usually provokes negative chronotropic and inotropic effects via activation of M_2 and, to a lesser degree, M_3 receptors (Dhein *et al.* 2001; Wang *et al.* 2007). Although the molecular mechanisms of its effects have been explored quite thoroughly, less is known about the mechanisms of secretion from parasympathetic nerve terminals.

It is known that the mediator may be released from the nerve terminal in quantal (Fatt & Katz, 1952) or non-quantal form (Katz & Miledi, 1977; Vyskocil & Illes, 1977). Both quantal and non-quantal mechanisms of release of ACh at the neuromuscular junction have been studied quite thoroughly. The microelectrode technique allows registration of miniature endplate potentials, which appear as a result of the spontaneous release of single quanta during resting conditions. Stimulation of motor axons leads to the synchronous release of several tens or hundreds of quanta and consequent multiquantal endplate potentials.

Non-quantal release of mediator occurs in resting conditions. The amplitude of membrane potential hyperpolarization (H-effect) produced by blocking the

DOI: 10.1113/expphysiol.2009.050302

postsynaptic nicotinic receptors using *d*-tubocurarine in muscle pretreated with an inhibitor of acetylcholinesterase (AChE) is used as an assay of non-quantal ACh release at the neuromuscular junction (Katz & Miledi, 1977; Vyskocil & Illes, 1977; Vyskocil *et al.* 1983).

The coexistence of the quantal and non-quantal types of secretion in the neuromuscular junction has been proved, and several important functions of non-quantal ACh release are known (Malomouzh & Nikolsky, 2007). The investigation of ACh release mechanisms in the heart is a much more difficult task because, in contrast to neuromuscular junction, ACh acts via metabotropic muscarinic receptors, and a small amount of ACh, such as a quantum, does not result in any changes of membrane potential. Therefore, the electrophysiological methods commonly used for investigation of synaptic processes in the neuromuscular junction are not suitable here. The most important indirect evidence of quantal release of ACh from parasympathetic nerve endings in the mammalian heart is the presence of small (40–50 nm) vesicles inside the special varicosities, which form synaptic junctions with cardiomyocytes (Choate *et al.* 1993). Nothing is known about non-quantal release of ACh in the myocardium. In our recent studies, we observed cardiotropic effects of AChE inhibitors that are very similar to the effects of exogenous ACh (Abramochkin *et al.* 2008, 2009). In the present study, we provide the first evidence of non-quantal secretion of ACh from the parasympathetic nerve terminals in the rat heart.

Methods

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and the experimental protocol was approved by the Bioethics Committee of Moscow State University and the Animal Care and Use Committee of Kazan State Medical University.

Intracellular recordings of spontaneous action potentials in isolated right atrium of rats

Male Wistar rats ($n = 97$) weighing 280–320 g were decapitated using a guillotine for small animals (OpenScience, Moscow, Russia), the chest was opened and the heart rapidly excised and immersed in a physiological solution containing (mM): NaCl, 130.0; KCl, 5.6; NaH_2PO_4, 0.6; $MgCl_2$, 1.1; $CaCl_2$, 1.8; $NaHCO_3$, 20.0 and glucose, 11.0, bubbled with carbogen (95% O_2–5% CO_2), with pH 7.4 ± 0.2. The right atrial preparation, including the auricle, the crista terminalis, the intercaval region and the sinoatrial node, was isolated and pinned to the bottom of an experimental chamber (3 ml) supplied with a physiological solution at 10 ml min^{-1} (37.5°C). After 2 h of equilibration, transmembrane potentials were recorded with glass microelectrodes (20–30 MΩ) filled with 3 M KCl. The signal was digitized and analysed using specific software (L-card, Moscow, Russia; Synaptosoft, Decatur, GA, USA). Spontaneously occurring action potentials (APs) were recorded from the endocardial surface of the auricle. Stable impalements were maintained during the entire period action of the drugs. Changes in the cycle length (CL) and the AP duration to 50 (APD_{50}) and 90% of repolarization (APD_{90}) were analysed.

Experiments with *Clostridium botulinum* toxin type A

It is known that *Chlostridium botulinum* toxin type A (BT) penetrates into the nerve ending via endocytosis and cleaves proteins of the SNARE complex, which maintains the exocytosis of synaptic vesicles. Thus, BT blocks the mechanism of quantal release (Bullens *et al.* 2002). To achieve full entry of BT into the nerve endings we have used an incubation protocol practically identical to that described by Bullens *et al.* (2002). After excision from the heart, the right atrial preparations were incubated in physiological solution containing BT (50 U ml^{-1}) for 4 h. During the incubation, continuous oxygenation of the solution was maintained, the temperature was 32°C, and field stimulation was applied to the preparation (1 Hz, 1 ms, 15 V) to provide better penetration of toxin into the nerve terminals. In the neuromuscular preparation (32°C), analogous incubation leads to full cessation of synaptic transmission. However, we could not maintain the flow of solution during the incubation because of the expense of BT. Therefore, the temperature was maintained at 32°C to improve preparation viability. We have also performed additional control experiments with similar incubation of preparations without BT.

Detection of endocytosis and exocytosis using fluorescent dye FM1-43

We used loading of autonomic intramural nerve fibres with the fluorescent dye FM1-43 (Molecular Probes, Eugene, OR, USA) as a marker of endocytosis. Endocytosis in the terminals of postganglionic neurons was detected, and its intensity was estimated by measurement of the fluorescence of vesicles, which arise as a result of endocytosis, stained with FM1-43 (2.5×10^{-6} M; Cochilla *et al.* 1999). To activate endocytosis, the stimulation of intramural nerves in the presence of FM1-43 was carried out via a well-established technique first described by Vincenzi & West (1963). The frequency of stimulation was 100 Hz, stimulus duration 100 μs, amplitude 15 V, duration of stimulus trains 15 s and duration of gaps between trains 45 s. Stimulation caused loading of nerve fibres with dye. The duration of the loading protocol was 20 min; this duration was selected in pilot experiments.

After the dye loading, FM1-43 was washed out from the preparation with physiological solution containing cyclodextrin ADVASEP-7 (2×10^{-4} M; Sigma, St Louis, MO, USA), which binds non-internalized molecules of dye and reduces background fluorescence (Cochilla *et al.* 1999). In several experiments, unloading of FM1-43 from the nerve fibres was provoked by field stimulation using similar protocol of duration 50 min. An Olympus BX51 fluorescence microscope with a ×40 water-immersion objective (Olympus, Tokyo, Japan) and 510 nm bandpass filter (Chroma, Rockingham, VT, USA) was used for visualization of nerve fibres loaded with FM1-43. Images were acquired with an AxioCam MRm camera (Zeiss, Jena, Germany). Exciting light (488 nm) was emitted with a monochromator Polychrome V (TILL Photonics, Munich, Germany). Analysis of fluorescence images was performed using ImageJ 1.42 software (freeware downloaded from NIH source http://rsb.info.nih.gov/ij/).

Chronic sympathectomy in rats

Experiments with sympathectomized animals were conducted to show the presence of endocytosis in cholinergic intramural nerves. It is known that administration of guanethidine to newborn rats destroys sympathetic neurons and blocks the development of the sympathetic nervous system (Johnson *et al.* 1975). Chronic sympathectomy was induced in neonatal Wistar rats by subcutaneous injections of guanethidine sulphate: 25 mg kg^{-1} daily on days 1–14 after birth and 50 mg kg^{-1} daily on days 15–42. Injections were performed 6 days per week. The guanethidine solution was prepared in saline (0.9% NaCl). Control rats received daily injections of the same volume of saline (1–2.5 μl (g body weight)$^{-1}$).

Drugs

Armin (diethoxy-*p*-nitrophenyl phosphate), an organophosphate inhibitor of AChE, was manufactured by the Institute of Organic Chemistry, Moscow, Russia. Neostigmine, a carbamate inhibitor of AChE, atropine, hexamethonium bromide, hemicholinium III, vesamicol, tetrodotoxin and guanethidine sulphate were purchased from Sigma (St Louis, MO, USA). *Clostridium botulinum* toxin type A was purchased from Ipsen (Slough, UK).

Data analysis

All results in the text, tables and figures are expressed as means ± S.E.M. for n experiments. All samples were tested with Kolmogorov–Smirnov normality test. Each sample differed significantly from the normal distribution ($P < 0.05$ for every sample), so we used non-parametric tests for analysis. The effects of armin or neostigmine alone or in the presence of other compounds on AP duration (APD) and CL were compared with respective basal values of APD and CL by Wilcoxon signed rank test. The effects of armin and neostigmine in the absence and presence of atropine were also compared by Wilcoxon signed rank test. The effects of different concentrations of armin were compared by Mann–Whitney U test, because we could test only one concentration of this irreversible inhibitor in each experiment. The effects of armin in the absence and presence of hexamethonium, hemicholinium III, vesamicol, tetrodotoxin and BT were compared by Mann–Whitney U test. The relative intensity of FM1-43 fluorescence during destaining in the different groups of preparations was also compared by Mann–Whitney U test. $P \leq 0.05$ was adopted as the level of significance.

Results

Effects of AChE inhibitors

In the initial stage of our study, we investigated the modulation of APD and CL during 15 min superfusion by solution containing armin in different concentrations. During the control conditions, APD_{50}, APD_{90} and CL were 19.3 ± 2.2, 53.8 ± 4.5 and 190.2 ± 14.0 ms, respectively. The application of armin (10^{-7}, 10^{-6} and 10^{-5} M) produced a marked decrease in APD_{50} and APD_{90} (Figs 1 and 2*A*) and an increase in CL (Fig. 1). These effects of armin were slowly developing, reaching the maximum after 12–13 min of superfusion. The time course of development of the effect was similar in all subsequent experiments with armin and neostigmine, so we discuss only the maximal values of AP shortening and prolongation of the CL. No significant changes of the resting membrane potential were registered in any of the electrophysiological experiments with AChE inhibitors.

It should be noticed that 10^{-5} M armin altered APD and CL less than 10^{-6} M. This difference may be due to the ACh

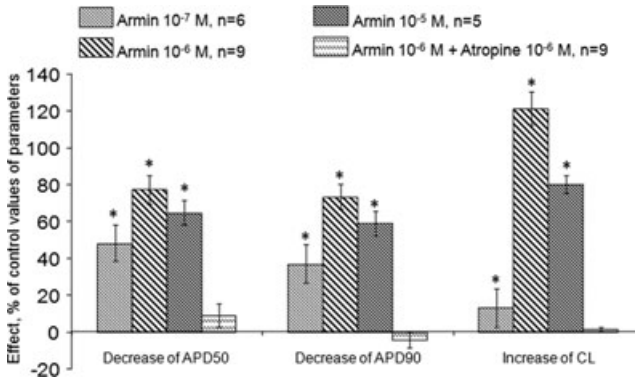

Figure 1. Effects of armin (10^{-7} M–10^{-5} M) on action potential duration (APD) and cycle length (CL), and the influence of 10^{-6} M atropine

Ordinates are percentage decrease in APD or percentage increase in CL. *$P < 0.05$ *versus* the respective control values.

receptor blocking action of high armin concentrations (Danilov & Ivanov, 1972).

We have recently demonstrated that application of neostigmine (10^{-7}, 10^{-6} and 5×10^{-6} M) produces an analogous concentration-dependent decrease of APD_{50} and APD_{90} and slowing of the sinus rate (Abramochkin *et al.* 2009).

Thus, organophosphate and carbamate AChE inhibitors caused monodirectional changes in the pattern of electrical activity that closely resemble typical effects of exogenous ACh, which are produced via activation of muscarinic receptors. To check the possible role of muscarinic receptors in the mediation of the effects of the AChE inhibitors, we applied atropine (10^{-6} M) in each experiment after 15 min of superfusion with 10^{-6} M armin. Atropine suppressed all effects of armin. It almost completely abolished the decrease in APD_{90} and increase in CL (Fig. 1) and also significantly reduced the decrease of APD_{50} induced by armin (Figs 1 and 2*A*). A similar action of atropine was shown in the neostigmine experiments (Abramochkin *et al.* 2009). Therefore, the effects of AChE inhibitors are ascribed to activation of muscarinic receptors induced by accumulation of ACh, but not to the additional effects of inhibitors.

These results raise the question of the mechanism of ACh release in the isolated atrium preparation, where vagal impulse activity is absent. Three ways of mediator secretion are known at present: evoked quantal release associated with excitation of the neuron, spontaneous quantal release and non-quantal release.

Investigation of the contribution of evoked quantal release to the accumulation of ACh in the myocardium during AChE inhibition

For many years, it was believed that parasympathetic efferent postganglionic neurons receive inputs only from preganglionic medullar parasympathetic neurons. From that point of view, the possibility of evoked quantal ACh release in our experiments seems negligible, because of the absence of impulse activity in preganglionic neurons. However, it is now proposed that afferent and local circuit neurons are also present in the heart and form a complex intracardiac nervous system with postganglionic efferent neurons (Armour, 1999). We may therefore propose impulse activity of postganglionic neurons even in the isolated atrium preparation. To check the possibility of the contribution of impulse activity to ACh release we have used two methods: blockade of AP generation with tetrodotoxin and blockade of ganglionic transmission with hexamethonium.

It is well known that tetrodotoxin blocks neuronal voltage-gated sodium channels in nanomolar concentrations, while sodium channels of the cardiac isoform $Na_v1.5$ are less sensitive and may be inhibited only by micromolar concentrations of tetrodotoxin (Goldin, 1999). That is why we decided to use tetrodotoxin (5×10^{-7} M) for blocking the putative AP generation in intracardiac nerve fibres. At this concentration it does not affect myocardial APs, while a lower concentration (3×10^{-7} M) was reported to block the fast sodium current in isolated and cultured neurons from rat parasympathetic intracardiac ganglia (Xu & Adams, 1992). We applied tetrodotoxin 10 min before and throughout the superfusion with armin (10^{-6} M). The effects of armin in the presence of tetrodotoxin did not differ significantly from the effects of armine alone (Fig. 3). However, we have also tried another method to suppress impulse activity of postganglionic neurons, namely blockade of ganglionic transmission.

It is accepted that ganglionic transmission in peripheral autonomic neurons is mediated by nicotinic receptors and can be blocked by their selective antagonist,

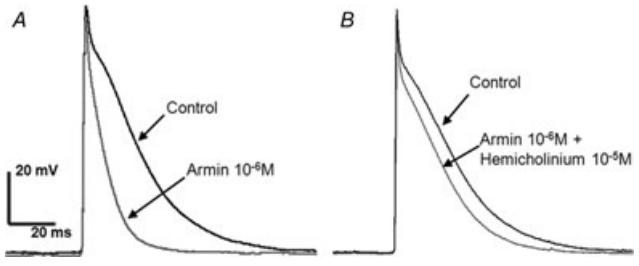

Figure 2. Changes in configuration of the action potential induced by 10^{-6} M armin alone (*A*) and 10^{-6} M armin in the presence of 10^{-5} M hemicholinium III (*B*)
Traces shown are original records from two separate experiments. *A*, membrane potential was −77.6 mV in control conditions and −78.9 mV in the presence of armin. *B*, membrane potential was −78.0 mV in control conditions and −78.7 mV in the combined presence of armin and hemicholinium III.

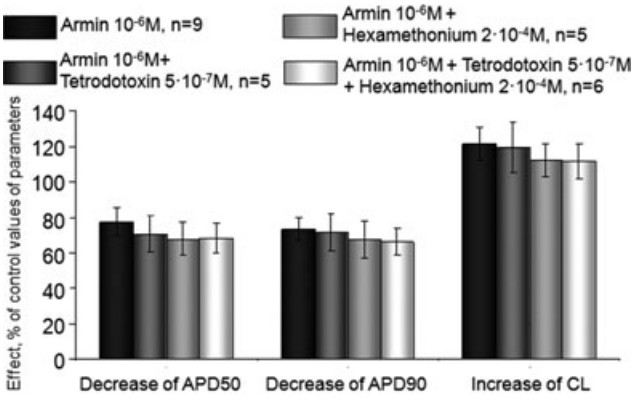

Figure 3. Effects of 10^{-6} M armin alone and in the presence of 5×10^{-5} M hexamethonium, 2×10^{-7} M tetrodotoxin and both these compounds on AP duration and CL
Ordinates are percentage decrease in APD or percentage increase in CL. For all columns $P < 0.05$ *versus* the respective control values.

hexamethonium bromide (Bibevski *et al.* 2000). In a special series of experiments ($n = 5$) we applied hexamethonium bromide (2×10^{-4} M) 10 min before and throughout the superfusion with armin (10^{-6} M). Shortening of AP and prolongation of the cycle length during superfusion with armin in the presence of hexamethonium bromide were slightly less prominent than during superfusion with armin alone, but this difference was not significant (Fig. 3). In addition, we have performed experiments ($n = 6$) with application of both tetrodotoxin (5×10^{-7} M) and hexamethonium bromide (2×10^{-4} M) 10 min before and throughout the superfusion with armin (10^{-6} M). The effects of armin did not differ significantly from those in control conditions (Fig. 3).

Thus, putative block of ganglionic transmission as well as direct block of neuronal impulse activity does not have an influence on the effect of AChE inhibitors and presumably on the quantity of accumulated ACh. Therefore, our attempts to suppress the impulse activity in postganglionic neurons did not affect secretion of ACh in the isolated atrium preparation. We may suppose that accumulation of ACh is not caused by evoked quantal release.

Effects of armin after the block of exocytosis with BT

It is known that all types of quantal secretion, including spontaneous quantal release of ACh, can be abolished by BT, a metalloprotease that cleaves synaptic proteins that mediate the transmitter release (SNARE complex; Kalandakanond & Coffield, 2001). We have used BT to block exocytosis in the right atrium preparation and thus exclude the possibility of quantal ACh release.

The comparison between effects of armin in preparations incubated in the presence of BT and without it is shown in Fig. 4. In both groups, the effects are less prominent than in fresh preparations (Fig. 1), presumably due to 4 h of incubation. We have failed to find a significant difference in the magnitude of APD decrease and increase of CL between the control and the experimental group of incubated preparations. Thus, blockade of both evoked and spontaneous quantal secretion by BT does not affect the accumulation of ACh in the myocardium during AChE inhibition.

Some questions concerning the effects of BT in our preparations may be raised. To get access to the SNARE complex, the BT should be taken up into parasympathetic endings by endocytosis. However, the processes of endo- and exocytosis in intramural cardiac nerves have scarcely been studied, so it was important to confirm the existence of endocytosis and the block of exocytosis by BT in our preparations.

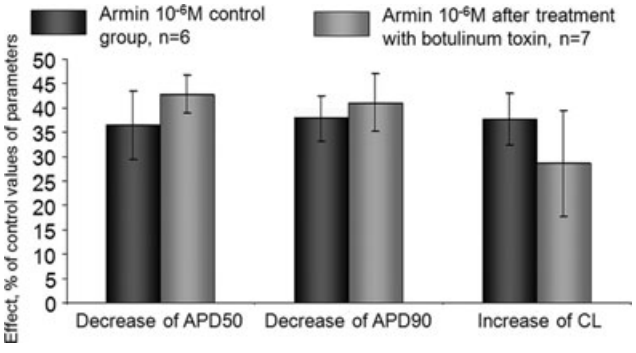

Figure 4. Comparison between effects of 10^{-6} M armin in preparations incubated for 4 h in a solution containing 50 U ml^{-1} botulinum toxin and without toxin
Ordinates are percentage decrease in APD or percentage increase in CL. For all columns $P < 0.05$ *versus* the respective control values.

Determination of endocytosis and exocytosis in the intramural nerve fibres of the rat right atrium

The presence of endocytosis was demonstrated in the postganglionic nerve fibres of rat right atrium by loading of nerve terminals with the fluorescent dye FM1-43, which binds with the cell membrane and therefore stains synaptic vesicles that arise due to endocytosis during the stimulation of nerves (Cochilla *et al.* 1999). Stimulation of the autonomic intramural nerves in the caudal part of the crista terminalis and intercaval region provoked the appearance of stained structures, which may be identified as intramural nerve fibres of the right atrium (Fig. 5*A*). The fibres were stained in a broken manner, with several points of intensive fluorescence along their length. In the absence of nerve stimulation, spontaneous loading of nerves with FM1-43 was not observed. Thus, an active process of endocytosis is present in our preparations of right atrium ($n = 8$). It is well known that parasympathetic and sympathetic postganglionic fibres coexist in the atrial myocardium. Therefore, experiments on sympathectomized rats, lacking postganglionic

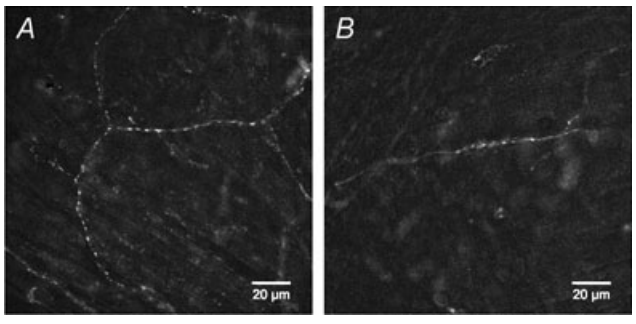

Figure 5. Sites of endocytosis, stained by FM1-43
A, fluorescence of intramural nerve fibres in the right atrium of a control rat. *B*, fluorescence of cholinergic intramural nerve fibres in the right atrium of a sympathectomized rat.

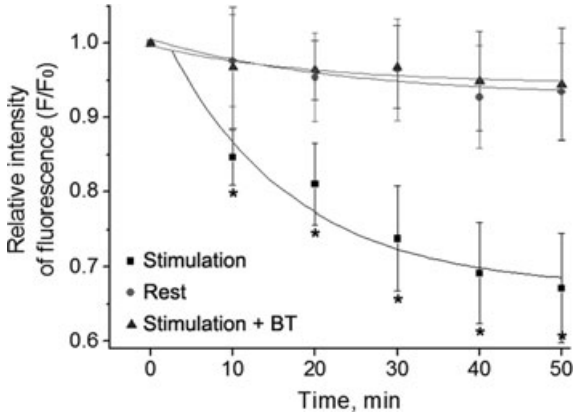

Figure 6. Decrease of relative fluorescence intensity in resting conditions and during unloading of FM1-43 from intramural nerve fibres in the presence of botulinum toxin (BT) and without it
*$P < 0.05$ *versus* stimulation in the presence of BT.

sympathetic fibres, were conducted to confirm the presence of endocytosis exactly in cholinergic fibres. In the right atrium from sympathectomized animals, stimulation of intramural fibres in the presence of FM1-43 also caused loading of nerve fibres with dye (Fig. 5*B*). Thus, endocytosis is present in the intramural cholinergic fibres and BT may be internalized effectively.

We have also performed experiments with unloading of FM1-43 from the nerve fibres in preparations incubated in the presence of BT ($n = 5$) and without it ($n = 5$). Additional experiments were conducted to observe destaining in resting conditions without stimulation in preparations incubated without BT ($n = 5$). Marked reduction of the fluorescence intensity was observed in the preparations incubated without toxin during the 50 min of stimulation protocol (Fig. 6). On the contrary, no significant difference was found between destaining in the preparations treated with toxin and destaining in the resting conditions. Therefore, BT completely blocks exocytosis of vesicles from the stained fibres.

Influence of hemicholinium III and vesamicol on effects of AChE inhibitors

Thus, we conclude that the effects of AChE inhibitors cannot be explained by the accumulation of 'quantal' ACh. To test the hypothesis concerning mediation of the effects of AChE inhibitors by 'non-quantal' ACh, we studied the effects of armin and neostigmine in the presence of inhibitors of putative 'non-quantal' ACh transporters. In our earlier study, we have demonstrated that non-quantal release of ACh from nerve terminals at the neuromuscular junction is mediated by a high-affinity choline uptake system (Nikolsky *et al.* 1991). We have studied the influence of an inhibitor of this transporter, hemicholinium III, on the effects of AChE inhibitors. Application of hemicholinium III was started 10 min before beginning superfusion with a solution containing both AChE inhibitor and hemicholinium III.

Hemicholinium III (10^{-5} M) significantly reduced the decrease of APD and increase of CL caused by armin and neostigmine (Figs 2*B* and 7). Hemicholinium III suppressed the decrease of APD_{50} caused by armin (10^{-6} M) by 62.9%, the decrease of APD_{90} by 55.1% and the increase of CL by 87.5%. It also suppressed the decrease

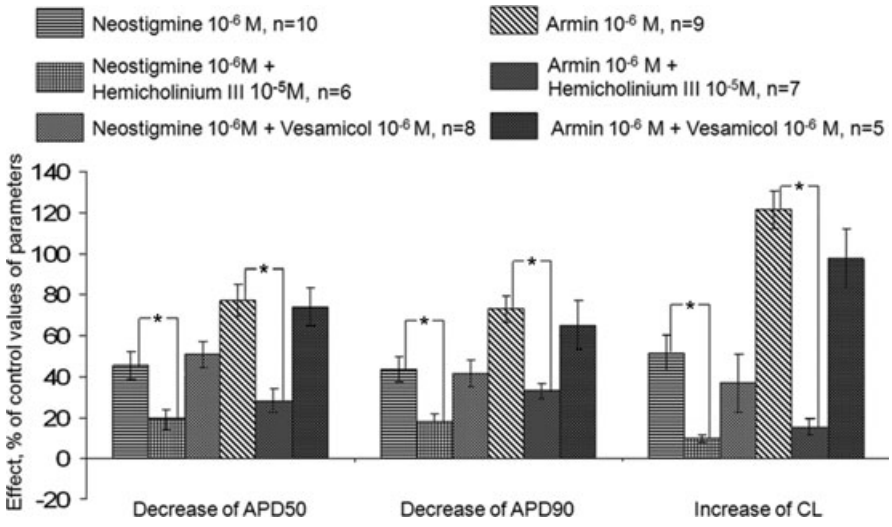

Figure 7. Effects of 10^{-6} M armin and 10^{-6} M neostigmine alone and in the presence of 10^{-5} M hemicholinium III or 10^{-6} M vesamicol on APD and CL
Ordinates are percentage decrease in APD or percentage increase in CL. For all columns, $P < 0.05$ *versus* the respective control values. *$P < 0.05$ *versus* armine or vesamicol alone.

of APD_{50} caused by neostigmine (5×10^{-6} M) by 58.3%, the decrease of APD_{90} by 59.1% and the increase of CL by 81.4%. The residual hemicholinium-resistant effect of neostigmine may be explained by its direct action as a muscarinic receptor agonist, shown earlier in the guinea-pig right atrium (Endou *et al.* 1997). However, the residual effects of armin require a different explanation, because armin never acts as a cholinomimetic and blocks muscarinic receptors in high concentrations ($>10^{-5}$ M; Danilov & Ivanov, 1972). Thus, the suppression of the effects of AChE inhibitors by hemicholinium III favours the hypothesis concerning non-quantal release of ACh in the myocardium.

However, hemicholinium III is a quaternary ammonium compound, and many such compounds may interact with muscarinic receptors. If hemicholinium III is capable of causing a partial blockade of muscarinic receptors, then the observed suppression of the effects of AChE inhibitors may be due to that side-effect of hemicholinium. To check this supposition, we studied the alteration of APD and CL by exogenous ACh (10^{-6} M) in the presence of hemicholinium III at 10^{-5} M (Fig. 8) and compared these effects with the action of ACh in control conditions. Hemicholinium III did not reduce the decrease of APD and increase of CL significantly in comparison to the effect of ACh in control conditions. This suggests that hemicholinium III does not block muscarinic receptors at the concentration that reduces the effects of AChE inhibitors. Thus, we may propose that the selective inhibitor of the high-affinity choline uptake system, hemicholinium III, suppresses the accumulation of ACh in the isolated rat atrium preparation. This observation supports the hypothesis concerning the non-quantal nature of ACh accumulation.

There is another hypothesis concerning the mechanisms of non-quantal secretion of ACh at the neuromuscular junction. According to this hypothesis, non-quantal release is mediated by the ACh transport system, which normally transports ACh into the synaptic vesicles (Edwards *et al.* 1985). The selective inhibitor of the ACh transport system, vesamicol, suppresses non-quantal release at the neuromuscular junction. Therefore, we performed experiments with vesamicol similar to the experiments with hemicholinium III. In these experiments, vesamicol (10^{-6} M) did not alter any effects of armin significantly (Fig. 7); however, at the neuromuscular junction this concentration of vesamicol is sufficient to suppress non-quantal ACh secretion. We conclude that the release of ACh in the atrial myocardium depends on the activity of the high-affinity choline uptake system, but not on the ACh transport system.

Thus, accumulation of ACh during inhibition of AChE is at least partly maintained by its non-quantal release from the parasympathetic nerve endings.

Discussion

Investigation of the mechanisms of ACh release in the myocardium is of considerable interest because of the important role of ACh in the regulation of heart function. However, common electrophysiological methods, used for analysis of synaptic processes at the neuromuscular junction, where ACh activates ionotropic nicotinic receptors and provokes generation of excitatory endplate potentials, are not suitable for the study of ACh release in the heart, because in the heart its effects are mediated via metabotropic muscarinic receptors. The discovery of vesicles in the autonomic postganglionic fibres of rat atrium, resembling the synaptic vesicles of motor nerve terminals (Choate *et al.* 1993), allows to propose that the mediator may be released in the form of quanta in the myocardium, as in the skeletal muscle. We have demonstrated that the inhibition of AChE exerts effects very similar to those of exogenous ACh. These effects are sensitive to atropine; therefore, we conclude that they are due to the accumulation of endogenous ACh. Although the leakage of ACh from parasympathetic fibres has been observed previously (Brown *et al.* 1982; Shimizu *et al.* 2009), we have tried to understand the nature of this phenomenon. It should be noted that experiments were carried out on isolated atrium in the absence of vagal impulse activity.

The results of experiments with tetrodotoxin and hexamethonium III seem to reject the hypothesis concerning accumulation of ACh as a result of impulse activity of postganglionic neurons arising in response to signals of intracardiac afferent neurons. However, we cannot be completely sure that the blockers were used in sufficiency high concentrations. Hexamethonium III at 10^{-3} M was reported to block ganglionic nicotinic receptors (Selyanko & Skok, 1992), while other authors

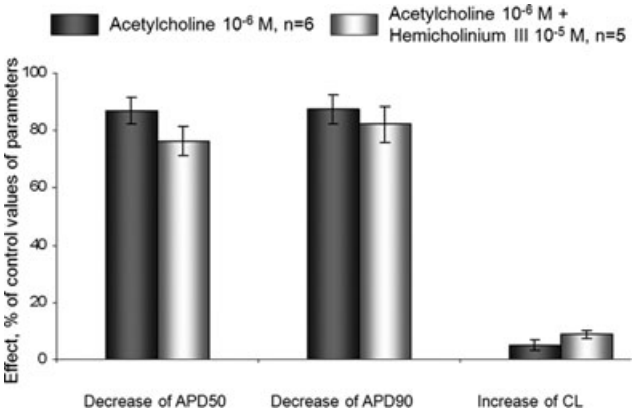

Figure 8. Effects of 10^{-6} M ACh in control conditions and in the presence of 10^{-5} M hemicholinium III
Ordinates are percentage decrease in APD or percentage increase in CL.

state that 10^{-4} M is absolutely sufficient (Xi-Moy *et al.* 1993). In contrast, hexamethonium at 10^{-4}–10^{-2} M acts as a muscarinic antagonist (Eglen *et al.* 1989). Therefore we used a compromise concentration, 2×10^{-4} M, which did not significantly suppress the effects of exogenous ACh in our pilot experiments. At a concentration of 3×10^{-7} M, tetrodotoxin was reported to block the fast sodium current in cultured neurons from rat parasympathetic intracardiac ganglia (Xu & Adams, 1992). However, there is evidence for a tetrodotoxin-resistant component in canine cardiac ganglion neurons (Scornik *et al.* 2006). Thus, we had to use additional methods to block evoked quantal release.

It could be proposed that accumulation of ACh during inhibition of AChE depends on spontaneous quantal release. Experiments with blockade of all forms of quantal release (evoked and spontaneous) by BT might finally clarify this question. Incubation of the isolated rat atrium preparation with BT at a concentration sufficient to abolish exocytosis at the neuromuscular junction did not alter the effects of AChE inhibitors. It is known that the blockade of exocytosis by BT is a result of its complex transformations inside the nerve ending: endocytosis of toxin and internalization into the endosomes, detachment of the light chain, its translocation to the cytosol and, finally, cleavage of the SNARE complex (Fujinaga, 2006). Therefore, we had to confirm that BT penetrates into the nerve endings and blocks exocytosis in our experimental conditions. In the experiments with the fluorescent dye FM1-43, we have demonstrated the presence of endocytosis in cholinergic fibres of the rat right atrium. Therefore, BT could enter the cholinergic varicosities. Moreover, BT completely blocked unloading of FM1-43 from the nerve fibres. We conclude that incubation with BT was sufficient to block the machinery of exocytosis. Thus, our data provide clear evidence that endogenous ACh, which accumulates during the inhibition of AChE, could not have a quantal nature.

This conclusion seems even more sensible when one considers that at the neuromuscular junction only 5% of ACh is released in the quantal form during resting conditions and the remaining 95% is released via non-quantal secretion (Katz & Miledi, 1977). Thus, it would appear that in the myocardium during resting conditions ACh is released from the parasympathetic postganglionic fibres in a non-quantal manner.

According to our previous study (Nikolsky *et al.* 1991), non-quantal release of ACh from motor nerve terminals is an active transport process, mediated by specific transporters, namely the high-affinity choline uptake system. It was proposed in the case of the neuromuscular junction that ACh is released non-quantally via choline transporters acting in reverse mode. Hemicholinium III rapidly blocks this pathway of ACh release and thus quickly stops non-quantal secretion directly, not via inhibition of ACh synthesis. This mechanism of non-quantal release has previously been demonstrated for glutamate (Szatkowski *et al.* 1990) and dopamine (Sulzer *et al.* 1993), although it is still not fully proven for ACh. In the present study, we have shown that hemicholinium III, the selective blocker of this transport system which abolishes non-quantal release at the neuromuscular junction, appreciably suppresses the effects of AChE inhibitors. This fact also confirms that background secretion of ACh in the isolated atrium preparation is caused preferentially by non-quantal release. However, the mechanism of this phenomenon in the heart needs to be evaluated in further studies.

What is the physiological role of non-quantal ACh in the myocardium? To speculate about its putative physiological contribution, we should understand the possible effects of constitutive exposure of the myocardium to low concentrations of ACh. An important cardioprotective role of the cholinergic influence mediated by M_2 and M_3 ACh receptors has been demonstrated in several studies *in vivo* (Rosenshtraukh *et al.* 1994) and *in vitro* (Yang *et al.* 2005; reviewed by Wang *et al.* 2007). Cardioprotective effects are mediated mainly via anti-apoptotic signalling pathways (Wang *et al.* 2007) and may not correlate with the muscarinic modulation of electrical activity (Rosenshtraukh *et al.* 1994). It is possible that non-quantally released ACh may be cardioprotective, although this point needs extensive further investigation.

References

Abramochkin DV, Nikol'skii EE & Rozenshtraukh LV (2008). Effects of inhibitors of acetylcholinesterase on the parameters of the electric activity of rat atrial myocardium. *Dokl Biol Sci* **420**, 1–4.

Abramochkin DV, Petrov KA, Zobov VV, Yagodina LO, Nikolsky EE & Rosenshtraukh LV (2009). Mechanisms of cardiac muscle insensitivity to a novel acetylcholinesterase inhibitor C-547. *J Cardiovasc Pharmacol* **53**, 162–166.

Armour JA (1999). Myocardial ischaemia and the cardiac nervous system. *Cardiovasc Res* **41**, 41–54.

Bibevski S., Zhou Y, McIntosh JM, Zigmond RE & Dunlap ME (2000). Functional nicotinic acetylcholine receptors that mediate ganglionic transmission in cardiac parasympathetic neurons. *J Neurosc* **20**, 5076–5082.

Brown JH, Wetzel GT & Dunlap J (1982). Activation and blockade of cardiac muscarinic receptors by endogenous acetylcholine and cholinesterase inhibitors. *J Pharmacol Exp Ther* **223**, 20–24.

Bullens RWM, O'Hanlon GM, Wagner E, Molenaar PC, Furukawa K, Furukawa K, Plomp JJ & Willison HJ (2002). Complex gangliosides at the neuromuscular junction are membrane receptors for autoantibodies and botulinum neurotoxin but redundant for normal synaptic function. *J Neurosc* **22**, 6876–6884.

Choate JK, Klemm M & Hirst GDS (1993). Sympathetic and parasympathetic neuromuscular junctions in the guinea-pig sino-atrial node. *J Auton Nerv Syst* **44**, 1–16.

Cochilla AJ, Angleson JK & Betz WJ (1999). Monitoring secretory membrane with FM1-43 fluorescence. *Annu Rev Neurosci* **22**, 1–10.

Danilov AF & Ivanov IuIa (1972). The mechanism of blockade and recovery of neuromuscular transmission under the influence of armin in experiments on cats. *Farmakol Toksikol* **35**, 571–575.

Dhein S, Van Koppen CJ & Brodde O (2001). Muscarinic receptors in the mammalian heart. *Pharmacol Res* **44**, 161–182.

Edwards C, Dolezal V, Tucek S, Zemková H & Vyskocil F (1985). Is an acetylcholine transport system responsible for nonquantal release of acetylcholine at the rodent myoneural junction? *Proc Natl Acad Sci USA* **82**, 3514–3518.

Eglen RM, Michel AD, Cornett CM, Kunysz EA & Whiting RL (1989). The interaction of hexamethonium with muscarinic receptor subtypes *in vitro*. *Br J Pharmacol* **98**, 499–506.

Endou M, Tanito Y & Okumura F (1997). A comparison between chronotropic effects of neostigmine and edrophonium in isolated guinea pig right atrium. *J Pharmacol Exp Ther* **282**, 1480–1486.

Fatt P & Katz B (1952). Spontaneous subthreshold activity at motor nerve endings. *J Physiol* **117**, 109–128.

Fujinaga Y (2006). Transport of bacterial toxins into target cells: pathways followed by cholera toxin and botulinum progenitor toxin. *J Biochem* **140**, 155–160.

Goldin AL (1999). Diversity of mammalian voltage-gated sodium channels. *Ann N Y Acad Sci* **868**, 38–50.

Johnson EM, Cantor E & Douglas JR (1975). Biochemical and functional evaluation of the sympathectomy produced by the administration of guanethidine to newborn rats. *J Pharmacol Exp Ther* **193**, 503–512.

Kalandakanond S & Coffield JA (2001). Cleavage of intracellular substrates of botulinum toxins A, C and D in a mammalian target tissue. *J Pharmacol Exp Ther* **296**, 749–755.

Katz B & Miledi R (1977). Transmitter leakage from motor nerve endings. *Proc R Soc Lond B Biol Sci* **196**, 59–72.

Malomouzh AI & Nikolsky EE (2007). Non-quantal secretion of acetylcholine from the motor nerve endings: molecular mechanism, physiological role, regulation. *Neurofiziologiya* **39**, 352–363.

Nikolsky EE, Voronin VA, Oranska TI & Vyscocil F (1991). The dependence of non-quantal acetylcholine release on the choline-uptake system in the mouse diaphragm. *Pflugers Arch* **418**, 74–78.

Rosenshtraukh L, Danilo P, Anyukhovsky EP, Steinberg SF, Rybin V, Brittain-Valenti K, Molina-Viamonte V & Rosen MR (1994). Mechanisms for vagal modulation of ventricular repolarization and of coronary occlusion-induced lethal arrhythmias in cats. *Circ Res* **74**, 722–732.

Scornik FS, Desai M, Brugada R, Guerchicoff A, Pollevick GD, Antzelevitch C & Perez GJ (2006). Functional expression of 'cardiac-type' Nav1.5 sodium channel in canine intracardiac ganglia. *Heart Rhythm* **3**, 842–850.

Selyanko AA & Skok VI (1992). Acetylcholine receptors in rat cardiac neurones. *J Auton Nerv Syst* **40**, 33–48.

Shimizu S, Akiyama T, Kawada T, Shishido T, Yamazaki T, Kamiya A, Mizuno M, Sano S & Sugimachi M (2009). In vivo direct monitoring of vagal acetylcholine release to the sinoatrial node. *Auton Neurosci* **148**, 44–49.

Sulzer D, Maidment NT & Rayport S (1993). Amphetamine and other weak bases act to promote reverse transport of dopamine in midbrain neurons. *J Neurochem* **60**, 527–535.

Szatkowski M, Barbour B & Attwell D (1990). Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* **348**, 443–446.

Vincenzi FF & West TC (1963). Release of autonomic mediators in cardiac tissue by direct subthreshold electrical stimulation. *J Pharmacol Exp Ther* **141**, 185–194.

Vyskocil F & Illés P (1977). Non-quantal release of transmitter at mouse neuromuscular junction and its dependence on the activity of Na^+-K^+ ATP-ase. *Pflugers Arch* **370**, 295–297.

Vyskocil F, Nikolsky E & Edwards C (1983). An analysis of the mechanisms underlying the non-quantal release of acetylcholine at the mouse neuromuscular junction. *Neuroscience* **9**, 429–435.

Wang H, Lu Y & Wang Z (2007). Function of cardiac M_3 receptors. *Auton Autacoid Pharmacol* **27**, 1–11.

Xi-Moy SX, Randall WC & Wurster RD (1993). Nicotinic and muscarinic synaptic transmission in canine intracardiac ganglion cells innervating the sinoatrial node. *J Auton Nerv Syst* **42**, 201–213.

Xu ZJ & Adams DJ (1992). Voltage-dependent sodium and calcium currents in cultured parasympathetic neurones from rat intracardiac ganglia. *J Physiol* **456**, 425–441.

Yang B, Lin HX & Xu CQ (2005). Choline produces cytoprotective effects against ischemic myocardial injuries: evidence for the role of cardiac M_3 subtype mascarinic acetylcholine receptors. *Cell Physiol Biochem* **16**, 163–174.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (08-04-00923, 09-04-01280) and the President of Russian Federation Scientific Schools (177.2008.4).