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ORIGINAL PAPER

Effects of acetylcholinesterase inhibitor paraoxon denote the possibility of non-quantal acetylcholine release in myocardium of different vertebrates

Denis V. Abramochkin · Anastasia A. Borodinova · Leonid V. Rosenshtraukh

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Abstract Effects of organophosphorous acetylcholinesterase inhibitor paraoxon were studied in the isolated atrial and ventricular myocardium preparations of a fish (cod), an amphibian (frog) and a mammal (rat) using the microelectrode technique. Incubation of isolated atrium with paraoxon (5×10^{-6}–5×10^{-5} M) caused significant reduction of action potential duration and marked slowing of sinus rhythm. These effects were abolished by muscarinic blocker atropine and therefore are caused by acetylcholine, which accumulates in the myocardium due to acetylcholinesterase inhibition even in the absence of vagal input. Hemicholinium III is a blocker of high affinity choline-uptake transporters, which are believed to mediate non-quantal release of acetylcholine from cholinergic terminals in different tissues. In the atrial myocardium of all the three studied species, hemicholinium III (10^{-5} M) significantly suppressed all the effects of paraoxon. Blocker of parasympathetic ganglionic transmission hexamethonium bromide (10^{-4} M) and inhibitor of vesicular acetylcholine transporters vesamicol (10^{-5} M) failed to attenuate paraoxon effects. Among ventricular myocardium preparations of three species paraoxon provoked marked cholinergic effects only in frog, hemicholinium III abolished these effects effectively. We conclude that paraoxon stops degradation of acetylcholine in the myocardium and helps to reveal the effects of acetylcholine, which is continuously secreted from the cholinergic nerves in non-quantal manner. Thus, non-quantal release of acetylcholine in the heart is not specific only for mammals, but is also present in the hearts of different vertebrates.

Keywords Acetylcholine · Paraoxon · Atrium · Heart · Action potential · Fish

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D. V. Abramochkin · A. A. Borodinova
Department of Human and Animal Physiology,
Moscow State University, Leninskiye Gory, 1,
12, Moscow, Russia

D. V. Abramochkin · L. V. Rosenshtraukh
Laboratory of Cardiac Electrophysiology,
Institute of Experimental Cardiology, 3rd Cherepkovskaya,
15A, Moscow, Russia

D. V. Abramochkin (✉)
prospect Vernadskogo, 13, 21, Moscow, Russia
e-mail: abram340@mail.ru

Introduction

Parasympathetic regulation is extremely important for normal cardiac functioning in mammals and other vertebrates. Its significance is especially high in fishes, because sympathetic innervation is sparse in fish hearts (Laurent et al. 1983). The main effector substance of cardiac parasympathetic nerves is acetylcholine (ACh), which is released in the myocardium from postganglionic parasympathetic intramural neurons. ACh usually provokes negative chronotropic and inotropic effects via activation of M2 and, to much lesser degree, M3 G-protein coupled receptors (Dhein et al. 2001; Wang et al. 2007). Therefore, it is widely recognized that cholinergic effects in myocardium are mediated via inhibition of adenylate cyclase and activation of potassium ACh-dependent current (I_{KACh}), however, little is known about the mechanisms of ACh release from the postganglionic neurons.

For many years, mechanisms of ACh release were studied mainly in the neuromuscular junction. It is known that ACh may be released from the motor nerve terminal in quantal (Fatt and Katz 1952) or non-quantal (Katz and

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Miledi 1977; Vyskocil and Illes 1977) form. While quantal release is based on the spontaneous or evoked exocytosis of vesicles containing ACh, non-quantal release occurs as a continuous leakage of mediator from the nerve ending. Investigation of ACh release mechanisms in the cardiac muscle is substantially more difficult, because in contrast to neuromuscular junction ACh acts via metabotropic muscarinic receptors and little quantity of ACh, such as a quantum, does not alter the membrane potential significantly. Recently, our group has demonstrated the presence of non-quantal ACh secretion in the rat atrial myocardium in addition to common vesicular release (Abramochkin et al. 2010). In this study, we used acetylcholinesterase (AChE) inhibitors neostigmine and armin to provide accumulation of non-quantal ACh in isolated myocardial preparations, which lack efferent input from the vagus nerve. Accumulating non-quantal ACh produced typical cholinergic effects very sensitive to hemicholinium III, a blocker of choline uptake transporters. These transporters are believed to mediate the non-quantal release in neuromuscular junction, while working in reverse mode and taking ACh out of the nerve terminal (Chávez et al. 2011; Vyskocil et al. 2009). On the other hand, effects of paraoxon were not altered by ganglionic blocker hexamethonium. Using organophosphorous AChE inhibitor paraoxon Chávez et al. (2011) demonstrated the presence of non-quantal ACh release in smooth muscles of guinea pig trachea. Moreover, the authors confirmed the critical dependence of this process on the activity of choline uptake transporters.

However, there is still no evidence of non-quantal ACh release in the myocardium of non-mammalian vertebrates. In the present study, we demonstrate effects of organophosphorous AChE inhibitor paraoxon on electrical activity of atrial and ventricular isolated myocardial preparations of a fish, an amphibian and a mammal. We show that inhibition of AChE induces cholinergic effects, which can be suppressed by hemicholinium III and therefore likely to be mediated by accumulation of non-quantal ACh.

Materials and methods

The experimental protocol was approved by the Bioethics Committee of Moscow State University.

Experiments with cod (*Gadus morhua* L.) were performed at the White Sea Biological Station of Moscow State University (Karelia, Russia). The fishes (190 ± 45 g, $n = 33$) were caught in the sea near the biological station and kept in a cage (0.5 m^3), submerged into the sea (10°C), for 1–2 before the experiment. Frogs (*Rana temporaria* L.) (29 ± 2.5 g, $n = 39$) were caught in a forest near Moscow during the end of autumn and kept in a terrarium (20°C) before the experiment. For experiments on mammalian myocardium, male 3-month-old Wistar rats ($n = 44$) weighing 280–320 g were used. The standard laboratory rat food and distilled water were given ad libitum.

The animals were decapitated, the chest was opened and the heart was rapidly removed and immersed in physiological solution for marine fishes (composition (mM): NaCl 150, KCl 5.14, $NaH_2PO_4 \cdot 2H_2O$ 1, $NaHCO_3$ 25, $MgSO_4 \cdot 7H_2O$ 1.8, $CaCl_2 \cdot 2H_2O$ 1.25, glucose 5; pH 7.55 ± 0.05) (Hoglund and Gesser 1987), or standard Ringer's solution for frog [composition (mM): NaCl 120.4, KCl 2.5, $NaH_2PO_4 \cdot 2H_2O$ 0.35, $NaHCO_3$ 25, $MgSO_4 \cdot 7H_2O$ 1.05, $CaCl_2 \cdot H_2O$ 1.35, glucose 10.7; pH 7.3 ± 0.1] (Salas et al. 2006) or Tyrode solution for mammals [composition (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; pH 7.4 ± 0.1]. All solutions were continuously bubbled with carbogen (95% O_2, 5% CO_2), pH was adjusted with 1 N NaOH and 1 N HCl. After isolation, the preparations were splayed open and pinned in the Sylgard-coated experimental chamber (3 ml volume) endocardial side up. Preparations were superfused with a constant flow of physiological saline (10 ml/min flow rate). The temperature was maintained at 10°C for cod myocardium, 20°C for frog and 37.5°C for rat myocardium.

In the experiments with fish, we used the isolated atrial preparations either containing the sinoatrial ring, where the pacemaker of the fish heart is located (Haverinen and Vornanen 2007; Lukyanov et al. 1986), or preparations without the sinoatrial ring for the experiments with paced rhythm. In the latter case, the pacing frequency was always set slightly higher than the frequency of spontaneous beats to avoid the possible extra excitations. Pacing stimuli were applied via a pair of silver Teflon-coated electrodes connected to DL360 stimulator (Neurobiolab, Moscow, Russia). The average spontaneous beating rate in cod atrial preparations was 1.02 ± 0.04 Hz, therefore in experiments with paced cod myocardium the pacing frequency was 1.1 Hz. Analogously, in frogs we used spontaneously beating preparations containing sinus venosus or paced atrial preparations without sinus venosus. The average spontaneous beating rate in frog atrial preparations was 0.93 ± 0.04 Hz, the pacing frequency was 1 Hz. In rats, all experiments were conducted on spontaneously beating right atrial preparations including the auricle, the crista terminalis, the intercaval region and the sinoatrial node. In all studied species, some experiments were carried out on ventricular myocardial preparations, which were paced with the same frequency as the corresponding atrial preparations. For rat right ventricular wall preparations, the pacing frequency was 6 Hz.

Intracellular recordings of electrical activity

The floating microelectrode technique, which was described earlier (Coraboeuf 1969), was used with some modifications for intracellular recordings of electrical activity in fish and frog myocardium. Transmembrane potentials were recorded with glass microelectrodes (20–30 MΩ) filled with 3 M KCl and connected to a high input impedance amplifier (Model 3100, A-M Systems, Carlsborg, WA, USA) using 50 μm tungsten wire. This flexible connection allowed the microelectrode to stay in the cell during contraction of the preparation. In experiments with rat myocardium, standard technique with rigid fixation of microelectrode was used. The signal was digitized, recorded and analyzed using specific software (L-card, Moscow, Russia; Synaptosoft, Fort Lee, NJ, USA). Spontaneously occurring APs were recorded from the endocardial surface of preparations. Changes in the AP duration at 50 and 90% of repolarization (APD50, APD90), cycle length (CL) and AP amplitude (APA) under the action of paraoxon were determined.

Drugs

Organophosphorous AChE inhibitor paraoxon, muscarinic blocker atropine, ganglionic blocker hexamethonium bromide, inhibitor of high-affinity choline uptake transporter hemicholinium III and inhibitor of vesicular ACh transporter vesamicol were purchased from Sigma (St. Louis, MO, USA).

Paraoxon was applied in different concentrations (10^{-6}–5×10^{-5} M) for 12 min, it was given only once in each preparation because it inhibits AChE in irreversible manner and washout is not possible. Atropine (3×10^{-6} M) was applied for 5 min after 12 min of superfusion with paraoxon solution. Hexamethonium bromide (10^{-4} M), hemicholinium III (10^{-5} M) and vesamicol (10^{-5} M) were applied for 10 min before paraoxon application without subsequent washout and during all superfusion with paraoxon.

Statistical analysis

All results in the text and figures are expressed as mean ± s.e.m. for n experiments. The effects of paraoxon alone or in the presence of other compounds on APD, CL and APA were compared with the respective basal values of these parameters by Wilcoxon test. The effects of paraoxon in the absence and presence of atropine, hexamethonium, vesamicol or hemicholinium were compared by Mann–Whitney test, because we could test only one concentration of this irreversible AChE inhibitor in each experiment. $p \leq 0.05$ was adopted as the level of significance.

Results

Effects of paraoxon in atrial myocardium

At first, we have tested different concentrations of paraoxon in spontaneously beating atrial preparations to determine the most efficient concentration for each species, which is sufficient for further investigation of paraoxon effects. Average values of APD50, APD90 and CL during control conditions are presented in the Table 1. In the rat atrial myocardium preparations application of paraoxon (1×10^{-7}–1×10^{-5} M) produced a marked decrease in APD50, APD90 and increase in CL (Fig. 1a). These effects of paraoxon were slowly developing, reaching the maximum after 8–9 min of superfusion. The time course of paraoxon effect developing was similar in all subsequent experiments with this AChE inhibitor, therefore we further discuss only the maximal values of AP shortening and the prolongation of the CL. It should be noticed, that paraoxon 10^{-5} M altered APD and CL less than 5×10^{-6} M. This difference may be due to the blocking of muscarinic receptors by high paraoxon concentrations, as far as AChE inhibitors may interact with cholinoreceptors (Howard and Pope 2002). We have selected 5×10^{-6} M concentration for further experiments with rat myocardium.

In experiments with cod and frog atrial myocardium, we have also observed shortening of AP and slowing of spontaneous rhythm under the influence of paraoxon (Fig. 1b, c). In contrast to the rat atrium, the latter effect was much more prominent than the former. Higher concentrations of paraoxon were required to induce substantial effects, comparable to those in rat myocardial (Fig. 1), therefore, 5×10^{-5} M was selected for further experiments with fish and amphibian myocardium.

It is well-known that changes in beating rate may substantially alter APD. Therefore, experiments with 5×10^{-6} or 5×10^{-5} M paraoxon were performed in cod and frog atrial myocardial preparations paced with fixed frequency. In these experiments, decrease of APD50 and APD90 was much greater than in spontaneously beating preparations (Figs. 2, 3). This difference may be explained

Table 1 Control values of main parameters of electrical activity in different preparations

Preparation	APD50 (ms)	APD90 (ms)	CL (ms)
Cod atrium	174 ± 13	228 ± 15	980 ± 37
Cod ventricle	256 ± 17	274 ± 12	–
Frog atrium	132 ± 13	192 ± 17	1,075 ± 44
Frog ventricle	253 ± 18	284 ± 11	–
Rat atrium	13.7 ± 2.6	34.8 ± 3.3	191 ± 11
Rat ventricle	18.4 ± 3.9	38.7 ± 4.7	–

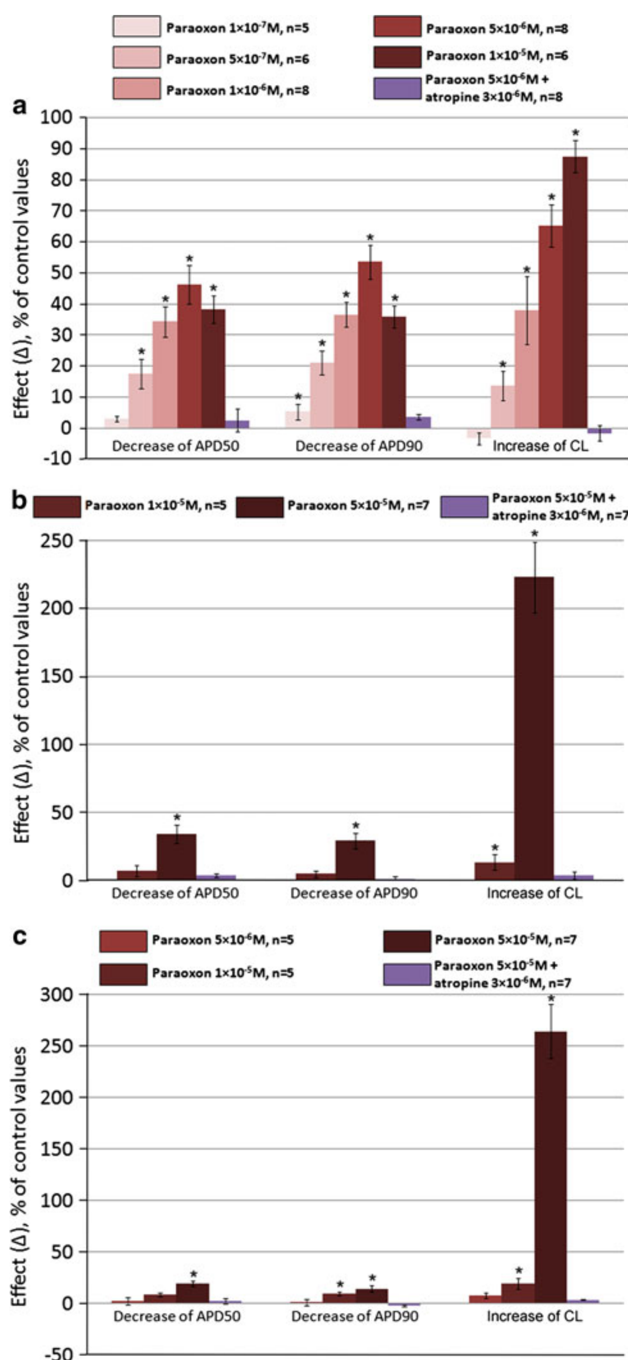

Fig. 1 Effects of paraoxon in different concentrations on AP duration and CL, and influence of 3×10^{-6} M atropine in spontaneously beating atrial preparations of rat (**a**), cod (**b**) and frog (**c**). Ordinates: % decrease in AP duration or % increase in CL. $*p < 0.05$ versus the respective control values

by the fact that slowing of heart rate itself leads to prolongation of AP. However, in our experiments shortening of AP induced by accumulating ACh was so prominent that the overall influence of paraoxon on APD was negative. It should be noted that among typical effects 5×10^{-5} M paraoxon produced slight reduction of APA in paced frog

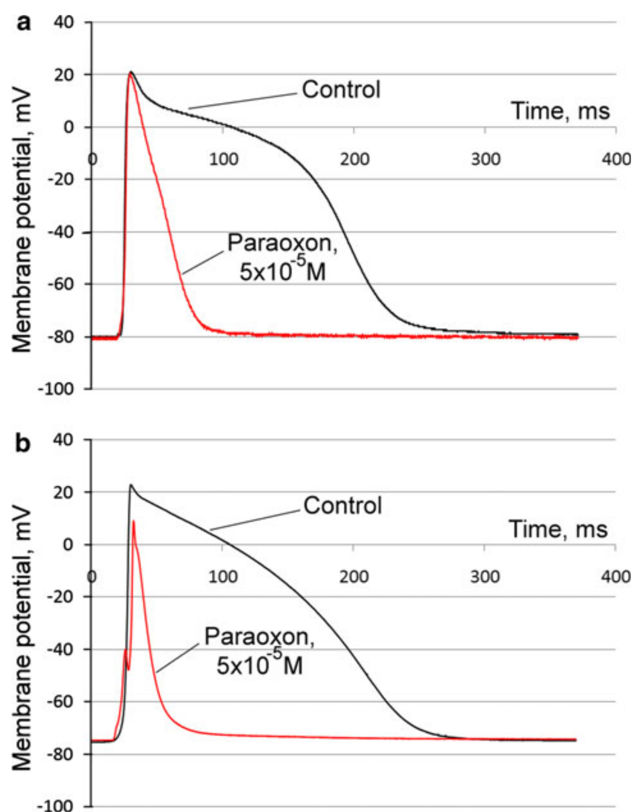

Fig. 2 Changes in configuration of AP induced by 5×10^{-5} M paraoxon in cod (**a**) and frog (**b**) paced atrial preparations (original records from two separate experiments)

atrial myocardium. Average decrease of APA was 13.38 ± 4.86% of control APA. In other experiments, paraoxon did not significantly alter APA. No significant changes of the resting membrane potential were registered in all experiments with paraoxon.

Thus, organophosphate AChE inhibitor paraoxon caused monodirectional changes in the pattern of electric activity in mammalian, fish and amphibian atrial myocardium 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 paraoxon effects we applied atropine (3×10^{-6} M) in each experiment after 12 min of superfusion with 5×10^{-6} or 5×10^{-5} M paraoxon. Atropine completely abolished all effects of paraoxon in all studied atrial preparations, both paced and spontaneously beating (Figs. 1, 3). Therefore, effects of paraoxon are ascribed to muscarinic receptors activation induced by accumulation of ACh in myocardium, but not to the additional non-cholinergic effects of inhibitors.

Effects of paraoxon in ventricular myocardium

In general, ventricular myocardium has lower density of parasympathetic innervations and is less sensitive to ACh

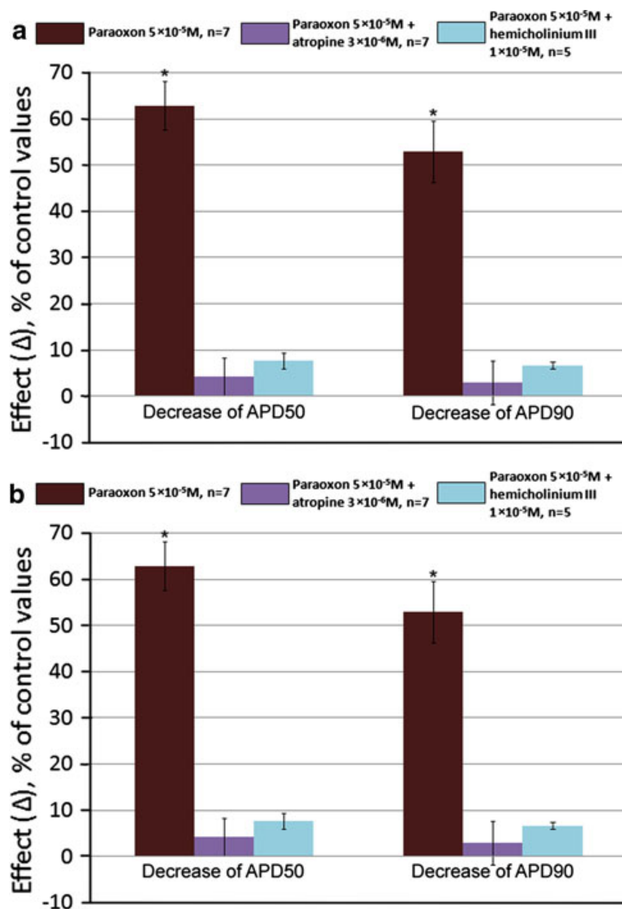

Fig. 3 Effects of 5×10^{-5} M paraoxon on AP duration in normal conditions and in the presence of 3×10^{-6} M atropine or 1×10^{-5} M hemicholinium III in paced atrial preparations of cod (**a**) and frog (**b**). Ordinates: % decrease in AP duration. $*p < 0.05$ versus the respective control values

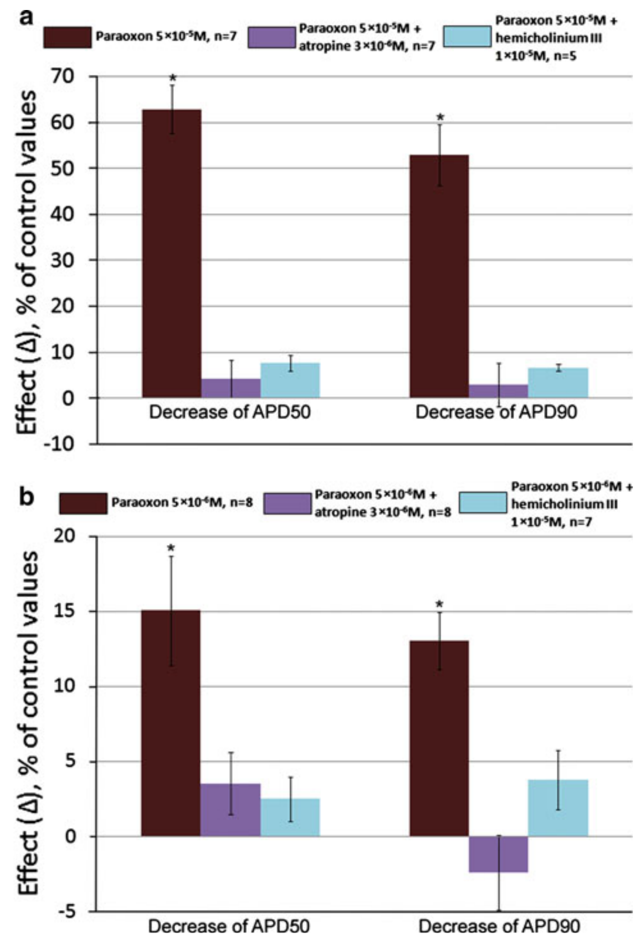

Fig. 4 Effects of paraoxon on AP duration in normal conditions and in the presence of 3×10^{-6} M atropine or 1×10^{-5} M hemicholinium III in paced ventricular preparations of frog (**a**) and rat (**b**). Ordinates: % decrease in AP duration. $*p < 0.05$ versus the respective control values

than atrial myocardium (Hoffman and Cranefield 1960), although there are several exceptions even among mammals (Abramochkin et al. 2006). Nevertheless, we studied effects of the most effective paraoxon concentrations (5×10^{-6} or 5×10^{-5} M) in paced ventricular myocardial preparations of cod, frog and rat. In cod ventricular myocardium, 5×10^{-5} M paraoxon did not induce significant change of AP waveform. This is not surprising in the light of several pilot experiments, which revealed complete insensitivity of cod ventricular myocardium to drastic concentration (10^{-4} M) of exogenous ACh.

However, in frog ventricular myocardium, we have observed typical effect of paraoxon: decrease of APD50 and APD90, which was completely abolished by 3×10^{-6} M atropine (Fig. 4). In rat, isolated right ventricular wall preparation, paraoxon, also induced atropine-sensitive shortening of AP. This effect was much smaller than in rat atrium, but significant.

Identification of endogenous ACh sources in isolated myocardium preparations

To find out whether ACh, which accumulates in the myocardium during AChE inhibition, is released in quantal or non-quantal form we performed experiments with ganglionic blocker hexamethonium bromide, inhibitor of vesicular ACh transporter vesamicol and inhibitor of choline uptake transporter hemicholinium III using atrial myocardium preparations.

Hexamethonium in high concentration (1×10^{-4} M) did not cause a significant change of paraoxon effects in atrial preparations of all studied species (Fig. 5). Vesamicol (1×10^{-5} M) also did not alter shortening of APs and slowing of rhythm induced by paraoxon. In contrast to these compounds, hemicholinium III significantly suppressed all effects of paraoxon in atria of all three species (Fig. 5). Action of hemicholinium was more prominent in frog and cod in comparison with rat. Similarly

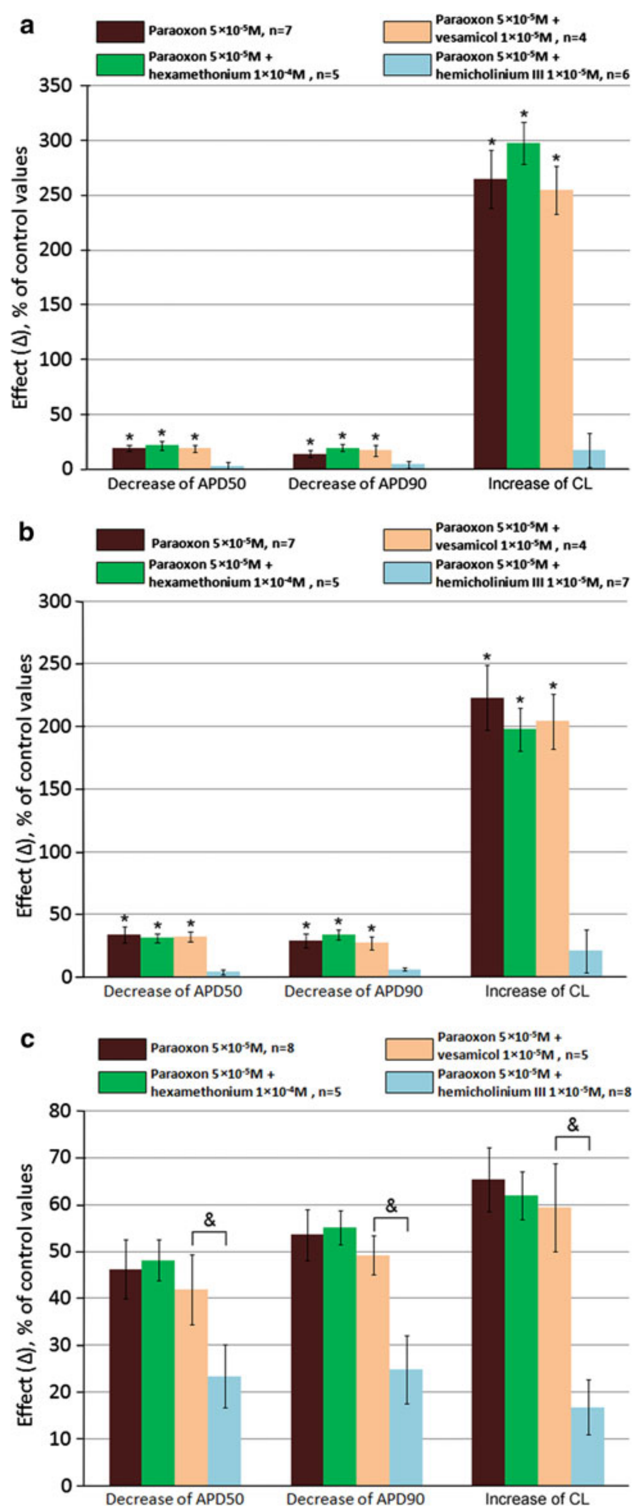

Fig. 5 Alteration of paraoxon effects by hexamethonium bromide (1×10^{-4} M), vesamicol (1×10^{-5} M) and hemicholinium III (1×10^{-5} M) in spontaneously beating atrial preparations from cod (**a**), frog (**b**) and rat (**c**). Ordinates: % decrease in AP duration or % increase in CL. $^{*}p < 0.05$ versus the respective control values. $^{\&}p < 0.05$ versus the effects of paraoxon alone, respectively

hemicholinium abolished the effects of paraoxon in paced atrial preparations of cod and frog (Fig. 3) and ventricular preparations of frog and rat (Fig. 4). In our previous study, we have assured that hemicholinium III does not block muscarinic receptors and its effects exclusively depend on inhibition of the choline uptake transporter. Thus, hemicholinium III suppresses effects of paraoxon in atrial and ventricular myocardium of cod, frog and rat via the inhibition of ACh accumulation.

Discussion

In the present study we provide the first, to our knowledge, demonstration of AChE inhibitor effects on electrical activity in the atrial myocardium of fish and compare it to the analogous effects of paraoxon in frog and rat myocardium. The full abolishment of paraoxon-induced AP shortening and slowing of spontaneous sinus rhythm by muscarinic blocker atropine clearly suggests the cholinergic nature of paraoxon effects. This fact is important in the light of several studies indicating the possibility of non-cholinergic effects of AChE inhibitors in the heart mediated by their direct binding to muscarinic receptors and insensitive to atropine (Silveira et al. 1990; Tryfonos et al. 2009).

Paraoxon belongs to the organophosphorous insecticides and is still used for agricultural purposes in third-world countries, although it can easily affect non-target organisms, especially aquatic organisms (Peter and Cherian 2000). Therefore, several attempts were made to reveal the mechanisms of paraoxon toxicity in fishes, particularly, its effects on contractility of fish atrial myocardium (Tryfonos et al. 2009). The authors showed the cholinergic nature of negative chronotropic and inotropic effects of paraoxon in the isolated spontaneously beating fish atrium. However, they did not explore the mechanism of endogenous ACh accumulation in isolated myocardium preparation where vagal impulse activity is absent. Our experiments confirm the assumption that this endogenous ACh may be released from the intracardiac parasympathetic nerves of fishes and amphibians in non-quantal form, as it was previously proved for mammals (Abramochkin et al. 2010).

Experiments with blocking of ganglionic synaptic nicotinic transmission by hexamethonium bromide, which did not alter the effects of paraoxon, allow us to exclude the possibility of putative spontaneous impulse activity of intracardiac nervous system, which may hypothetically induce evoked quantal release of ACh. However, the strongest evidence of non-quantal ACh release is suppression of paraoxon effects by hemicholinium III. It is accepted that ACh may be secreted from the nerve terminal in non-quantal form via vesicular ACh transporters, which

are build in the presynaptic membrane during exocytosis of synaptic vesicles, and high-affinity choline uptake transporters working in the reverse mode and pumping ACh outside the terminal (Vyskocil et al. 2009). The latter mechanism is confirmed by blocking of non-quantal ACh release with hemicholinium III and seems to be the only one present in the mammalian heart (Abramochkin et al. 2010) and smooth muscle (Chávez et al. 2011), because vesamicol does not alter effects of paraoxon. Moreover, Chávez et al. have elegantly showed that hemicholinium III abolishes non-quantal release via inhibition of choline uptake transporter per se, but not via reduction in the availability of choline for ACh synthesis. Therefore, hemicholinium III may be recognized as a specific blocker of non-quantal ACh release from cholinergic neurons. In our experiments, hemicholinium III caused partial, but substantial, suppression of paraoxon effects in the rat myocardium and almost completely blocked development of paraoxon effects in fish and frog. Therefore, we suppose that paraoxon induces accumulation of ACh in myocardium of fishes and amphibians, which is released in non-quantal form, as it was shown in mammals.

In the last years, several groups of researchers have found that ACh may be synthesised in non-neuronal tissues (Kawashima and Fujii 2008). In particular, mammalian cardiomyocytes are able to produce (Kakinuma et al. 2009) and release ACh (Rana et al. 2010). In non-neuronal tissues, the mechanism by which ACh is released involves activity of organic cation transporters, which are low-affinity, Na^+-independent transporters sensitive to quinine and corticosterone (Wessler et al. 2001; Wessler and Kirkpatrick 2001). These results beg the question of what is the source of non-quantal ACh in the myocardium of studied species. Is it released from postganglionic parasympathetic neurons or from cardiomyocytes? The fact that hemicholinium III, which selectively inhibits choline uptake transporters present exclusively in neurons (Yamamura and Snyder 1972; Guyenet et al. 1973), completely suppresses effects of paraoxon in myocardium of cod and frog clearly confirms the neuronal nature of non-quantal ACh in these species. On the contrary, in mammals the possibility of non-neuronal source of accumulating ACh cannot be excluded, because hemicholinium III reduces effects of paraoxon only partially.

However, our study has several significant limitations. First, in spite of strong certainty about non-quantal nature of ACh, which accumulates in the presence of AChE inhibitor in cod and frog myocardium, experiments with botulinic toxin, which cleaves proteins of SNARE complex and blocks any types of quantal neurotransmitter release, are needed for complete proof of these findings. Unfortunately, such experiments seem to be inadequate in fishes and amphibians, because the effect of botulinic toxin is a result of its complex transformations inside the nerve ending (Fujinaga 2006) and its effectiveness in lower vertebrates should be confirmed in dedicated studies. Second, we cannot reject the possible involvement of preganglionic intracardiac parasympathetic fibers in release of ACh in non-quantal form, although their density in the myocardium is much lower than the density of postganglionic cholinergic fibers and therefore their impact in overall cardiac ACh secretion seems negligible. Unfortunately, we are still far from complete understanding of mechanism of non-quantal ACh release via choline uptake transporters. It is hypothesized that transporters work in reverse mode if concentration of ACh inside the presynapse is high, while there is low concentration of choline in the synaptic cleft (Vyskocil et al. 2009). The intensity of non-quantal ACh release in the neuromuscular junction is controlled by different factors that induce nitric synthesis within the postsynaptic muscle fibers (Vyskocil et al. 2009). However, there is still no evidence of non-quantal release regulation in the heart. Finally, we are still far from understanding the physiological role of non-quantal ACh release both in mammalian and non-mammalian myocardium, although trophic or anti-apoptotic function of non-quantal ACh may be hypothesized since cardioprotective effects of ACh are well-known (Wang et al. 2007; Rosenshtraukh et al. 1994). There is also a possibility that non-quantal ACh release is associated only with the isolated myocardium and is absent in intact heart, although this phenomenon was confirmed for neuromuscular junction by in vivo experiments (Vyskocil et al. 2009).

In summary, our study demonstrates effects of organophosphorous AChE inhibitor paraoxon on electrical activity in the myocardium of fishes and providing significant evidence of non-quantal ACh release from intracardiac parasympathetic nerve fibers in non-mammalian vertebrates. We hope that this report will encourage further research of physiological role and regulatory mechanisms of this phenomenon.

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Conflict of interest There is no conflict of interests.

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