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Nicotinic potentiation of frog retinotectal transmission in tectum layer F by α3β2, α4β2, α2β4, α6β2, or α7 acetylcholine receptor subtypes

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ABSTRACT

Objective: The aim of the study was to explore the effect of semi-specific antagonists and agonists of the nicotinic acetylcholine receptors on the paired-pulse facilitation and nicotinic tonic and phasic potentiation of the frog retinotectal synaptic transmission.
Materials and methods: The experiments were performed *in vivo* on adult frogs, *Rana temporaria*. An individual retina ganglion cell (or its retinotectal fiber) was stimulated by current pulses delivered through multichannel stimulating electrode positioned on the retina. Responses to a discharge of a single retinal ganglion cell were recorded in the tectum by an extracellular carbon-fiber microelectrode positioned in the terminal arborization of the retinotectal fiber in the tectum layer F. The effect of the antagonists and agonists of the nicotinic acetylcholine receptors on the tectal responses has been tested.
Results: We found that the antagonists, MLA and DHβE, and agonists, RJR-2403 and choline, of the nicotinic acetylcholine receptors of the α3β2, α4β2, α2β4, α6β2 or α7 subtypes have had no effect on the phasic and tonic potentiation of the retinotectal transmission. The paired-pulse facilitation of the retinotectal transmission was not appreciably affected by the antagonists, but the choline, agonist of the α7 subtype receptor, has significantly decreased the paired-pulse facilitation.
Conclusions: The tonic and phasic potentiation of the retinotectal transmission in the tectum layer F were not mediated by the receptors of α3β2, α4β2, α2β4, α6β2 or α7 subtype. The results suggest that presynaptic nicotinic acetylcholine receptors of the frog optic fibers are different from those of the mammalian optic fibers.

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1. Introduction

Nicotinic acetylcholine receptors have pentameric structures, which are homomeric or heteromeric combinations composed of α (α2–α10) and β (β2–β4) subunits. They have different pharmacological and physiological properties based on the subunit composition. Both the heteromeric and homomeric nicotinic receptors are abundantly distributed in the CNS [1]. Predominant role of the CNS nicotinic acetylcholine receptors is thought to be in the presynaptic modulation of the synaptic transmission. Activation of the presynaptic nicotinic receptors leads to an increase of the release probability of various neurotransmitters [2,3].

Nicotinic acetylcholine receptors have been identified in the frog tectum by using immunocytochemical [4,5] and autoradiographic [6] techniques. The majority of non-α7 receptors are likely associated with retinal ganglion cell axon (optic fiber) terminals, whereas α7-containing receptors appear to have a different localization: on the terminals of the afferents from the nucleus isthmi and on the terminals of certain retina ganglion cells axons [6]. Most of the acetylcholine released in the frog tectum is synthesized by the nucleus isthmi receiving input from the optic tectum and sending cholinergic axons back to the optic tectum [7–9]. Direct stimulation of the nucleus isthmi enhances calcium influx into optic nerve fiber terminals in *Rana pipiens* [10]. Activation of the presynaptic nicotinic acetylcholine receptors causes the potentiation of glutamate release from the retinotectal synapses [5,11,12], and promotes calcium influx into the optic fiber terminals [13].

In our previous studies [11,12] we have demonstrated the phenomena of tonic and phasic (after-burst) nicotinic potentiation of a frog retinotectal synaptic transmission from retina afferents to the tectum layer F. In these studies it was shown, by the application of the nicotinic acetylcholine receptor antagonist d-tubocurarine, that these phenomena are generated through the activation of the presynaptic nicotinic acetylcholine receptors. Moreover, the receptors responsible for the tonic potentiation show high affinity, while the receptors responsible for the phasic potentiation – relatively low affinity to the acetylcholine. In the studies of Gotti et al. [14], Cox et al. [15], Mackey et al. [16], by using radioligand binding, immunoprecipitation and other biochemical methods and transgenic mice technique, have been demonstrated that the retina afferents to the rat superior colliculus possess nicotinic acetylcholine receptors of the α6β2*, α4α6β2*, α3β2* subtypes, and the afferents to the lateral geniculate nucleus possess the α4α6β2*, α6β2*, α4β2*, α2α6β2*, α3β2* subtypes. Since the superior colliculus of mammalian brain is the structure analogous to the optic tectum of the cold-blooded animals we expected that such subtypes of the nicotinic acetylcholine receptors may mediate the tonic and phasic potentiation of the retinotectal synaptic transmission to the frog tectum layer F. The α3β2, α4β2, or α2β4 receptors, due to their high affinity to the acetylcholine [1], would suit for the tonic potentiation, and the α6β2 or α7 receptors, due to their relatively low affinity to the acetylcholine, would suit for the phasic potentiation. In the present study we have applied the nicotinic acetylcholine receptor antagonists, methyllycaconitine (MLA) and dihydro-β-erythroidine (DHβE), and agonists, (E)-N-methyl-4-(3-pyridinyl)-3-butene-1-amine (RJR-2403) and choline to test our supposition.

2. Materials and methods

The experimental procedure has been described in detail in our earlier papers [11]. Here, we shortly emphasize the main points.

Experiments were performed *in vivo* on the adult frogs, *Rana temporaria*. All experiments in this study were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23 revised in 1985) and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the Animal Care and Use Committee of the State Food and Veterinary Service of Lithuania (No. 0237). During the surgical manipulations, frogs were anesthetized with high concentration of CO_2. The dorsal tectum was exposed in the manner described by Maturana et al. [17]. The retina, contralateral to the opened tectum, was prepared in the way described by George and Marks [18]. The eyeball cavity was filled, and the exposed dorsal tectum was perfused with Ringer's solution (in mM: 116 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 1.2 $NaHCO_3$, and 0.17 $NaH_2PO_4{\cdot}2H_2O$; pH 7.3–7.4). Frogs were immobilized using an intramuscular injection of 0.15–0.3 mg of d-tubocurarine, intubated and ventilated by a mechanical ventilator with a tidal volume of 2–3 mL at a frequency of 6–10 breaths per minute. Subsequent injections of 0.05–0.1 mg of d-tubocurarine were applied every hour to keep the frog immobilized during the experiment. Frogs were slightly anesthetized by placing them into 50 mg/L concentration solution of MS-222. All recordings were carried out in the dark at ambient temperatures of 17–22 °C. At the end of the experiments the animals were sacrificed with an anesthetic overdose.

An 8-channel electrode was used for stimulation of the retina. The electrode was made of eight 40 μm diameter tungsten wires (channels) bunched at 50–150 μm distances between centers of different wires. The stimulating electrode was placed on the nasoventral quadrant of the naked retina. Single and double current pulses of magnitude of 13–48 μA and duration of 50 μs, or a train of 8 of such pulses were applied to the retina through a pair of stimulating electrode channels using World Precision Instruments' isolator. The excitation of a single ganglion cell or its axon was achieved by switching between 8 channels of the electrode, changing the strength of the current pulse and, sometimes, slightly moving the stimulating electrode over the retina. Responses evoked in the F layer of the tectum by firing of a single retina ganglion cell (individual or unit responses) were recorded using carbon-fiber microelectrode positioned in the F layer of the tectum. Bare tip of the recording electrode was 50–70 μm long.

Solutions of the nicotinic acetylcholine receptor antagonists, methyllycaconitine (MLA, 1 μM) and dihydro-β-erythroidine (DHβE, 30 μM), and the agonists, (E)-N-methyl-4-(3-pyridinyl)-3-butene-1-amine (RJR-2403, 100 μM) and choline (5 mM) were prepared just before the use. The substances MLA and RJR-2403 were dissolved in the distilled water to get concentrated

solutions of 500 μM and 1 mM, respectively. The solutions were kept frozen. They were thawed out before the experiment, and the relevant amount of the concentrated solution was added into the vial with Ringer solution used to perfuse the frog brain surface to get the required concentration of the substance in the perfusion solution. Other two substances, DHβE and choline, were dissolved directly into the Ringer solution just before the experiment to get the relevant concentrations of the substances in the perfusion solution. The surface of the frog tectum was perfused with this solution at the rate of 0.4 mL/min. The perfusion lasted ~35 min. For most of the substances tested in our previous experiments such a duration was far enough to develop their effects. The chemicals were purchased from Sigma–Aldrich Co.

Averaged values are given as a mean ± SE (standard error of the mean). Paired *t*-test with confidence level of 0.95 was performed for estimation of statistical significance of the results.

3. Results

Recordings of the unit responses from individual retinotectal fiber terminals projecting to the F layer of the tectum have been achieved. The effect of the nicotinic acetylcholine receptor antagonists, MLA and DHβE, and agonists, RJR-2403 and choline, on the tonic potentiation, phasic potentiation and paired-pulse facilitation of the retinotectal synaptic transmission has been tested. The MLA and DHβE inhibit a range of nicotinic acetylcholine receptors, α3β2, α4β2, α2β4, α6β2, α7 subtypes among them [1]. The RJR-2403 is agonist with a preference to the α2β4 subtype. The choline is agonist with a preference to the α7 subtype.

The experiments were done as follows. The pair of current pulses at interpulse interval of 15 ms has been delivered and the response to the stimulus recorded (Fig. 1(a)). The amplitudes of the first and second fast synaptic potential (fSP) of the response, A_{fSP1} and A_{fSP2}, have been measured. The paired-pulse facilitation of the retinotectal transmission, f, has been calculated as a ratio of the amplitudes, A_{fSP2}/A_{fSP1}. The amplitude A_{fSP1} is tonic-potentiated by a factor of ~1.5 due to the ambient level of acetylcholine in the frog brain, as it was demonstrated in our earlier studies [11,12]. So, A_{fSP1} can be used as an indicative of the tonic potentiation of the retinotectal transmission. Next, the conditioning stimulus consisting of a train of 8 current pulses with interpulse intervals of 10 ms has been applied (Fig. 1(b)) to induce the phasic nicotinic potentiation of the retinotectal transmission. The amplitude of the first fSP of the response, A_{fSP}, has been measured. Then, the testing paired-pulse stimulation has been delivered 10 s after the delivery of the conditioning stimulus (Fig. 1(c)). The amplitude of the phasic-potentiated fSP, $A_{fSP,pot}$, has been measured. The phasic potentiation of the retinotectal transmission, P_{ph}, has been calculated as a ratio of the amplitudes of testing and conditioning fSPs, $A_{fSP,pot}/A_{fSP}$. The above sequence of stimuli has been delivered in the control conditions and during the application of the antagonists and agonists of the nicotinic acetylcholine receptors.

A total of 7 (with 5 frogs) and 10 (with 9 frogs) experiments were performed with the application of the antagonists DHβE (30 μM) and MLA (1 μM), respectively. The results are shown in Figs. 1 and 2. No significant effect on the paired-pulse facilitation, tonic and phasic potentiation has been detected: The $f = 1.3 \pm 0.07$, $A_{fSP1} = 119 \pm 11$ μV, $P_{ph} = 2.11 \pm 0.1$ in the control recordings, and $f = 1.3 \pm 0.07$, $n = 7$, $P = 1$, $A_{fSP1} = 121 \pm 12$ μV, $n = 7$, $P = 0.75$, $P_{ph} = 2.17 \pm 0.14$, $n = 7$, $P = 0.6$ when DHβE (30 μM) was present. The $f = 1.27 \pm 0.06$, $A_{fSP1} = 113 \pm 11$ μV, $P_{ph} = 2.18 \pm 0.1$ in the control recordings, and $f = 1.24 \pm 0.05$, $n = 10$, $P = 0.5$, $A_{fSP1} = 114 \pm 10$ μV, $n = 10$, $P = 1$, $P_{ph} = 2.07 \pm 0.09$, $n = 10$, $P = 0.2$ when MLA (1 μM) was present.

Six (with 4 frogs) and eight (with 6 frogs) experiments were performed with the application of the agonists RJR-2403 (100 μM) and choline (5 mM), respectively. The results are shown in Figs. 3 and 4. No significant effect on the paired-pulse facilitation, tonic and phasic potentiation has been detected in the case of the agonist RJR-2403: The $f = 1.33 \pm 0.06$, $A_{fSP1} = 108 \pm 20$ μV, $P_{ph} = 2.47 \pm 0.21$ in the control recordings, and $f = 1.22 \pm 0.03$, $n = 6$, $P = 0.1$, $A_{fSP1} = 123 \pm 16$ μV, $n = 6$, $P = 0.09$, $P_{ph} = 2.3 \pm 0.15$, $n = 6$, $P = 0.2$ when RJR-2403 (100 μM) was present. Choline has not had significant effect on the tonic and phasic potentiation and slightly but significantly decreased the paired-pulse facilitation: The $f = 1.19 \pm 0.05$, $A_{fSP1} = 125 \pm 16$ μV, $P_{ph} = 2.24 \pm 0.06$ in the control recordings, and $f = 1.125 \pm 0.045$, $n = 8$, $P = 0.01$, $A_{fSP1} = 140 \pm 20$ μV, $n = 8$, $P = 0.08$, $P_{ph} = 2.18 \pm 0.08$, $n = 8$, $P = 0.4$ when choline (5 mM) was present.

4. Discussion

The results of our earlier studies [11,12,19] have demonstrated that an application of carbamylcholine chloride (CCh), the non-specific acetylcholine receptor agonist, led to a 2–3 fold increase (potentiation) of the retinotectal synaptic transmission. Subsequent application of d-tubocurarine has eliminated this increase. An application of pilocarpine and oxotremorine-M, the specific muscarinic acetylcholine receptor agonists, had no effect on the retinotectal synaptic transmission. These results show that d-tubocurarine acts on the nicotinic acetylcholine receptors, not on the 5-HT3 and/or GABAA receptors [20,21]. The localization of these nicotinic acetylcholine receptors was of the presynaptic origin since the effect of d-tubocurarine was accompanied by a change (increase) in the paired-pulse facilitation. Thus, our previous studies have demonstrated that the tonic and phasic potentiation of the frog retinotectal transmission to the tectum layer F are mediated by presynaptic nicotinic acetylcholine receptors. In the present study we have tried to determine the subtypes of these nicotinic acetylcholine receptors by using of the semi-specific nAChR antagonists (MLA and DHβE) and agonists (RJR-2403, choline).

The value of the paired-pulse facilitation, f, would change compared to the control recordings if an antagonist or agonist had some presynaptic effect. If the tonic potentiation of the retinotectal transmission was mediated by the nicotinic acetylcholine receptors of the subtypes under consideration, the antagonists of those receptors would lead to a decrease of the A_{fSP1}, and agonists – to an increase or no change (in the case of saturation of the receptors by the ambient acetylcholine). If the phasic potentiation was mediated by the nicotinic

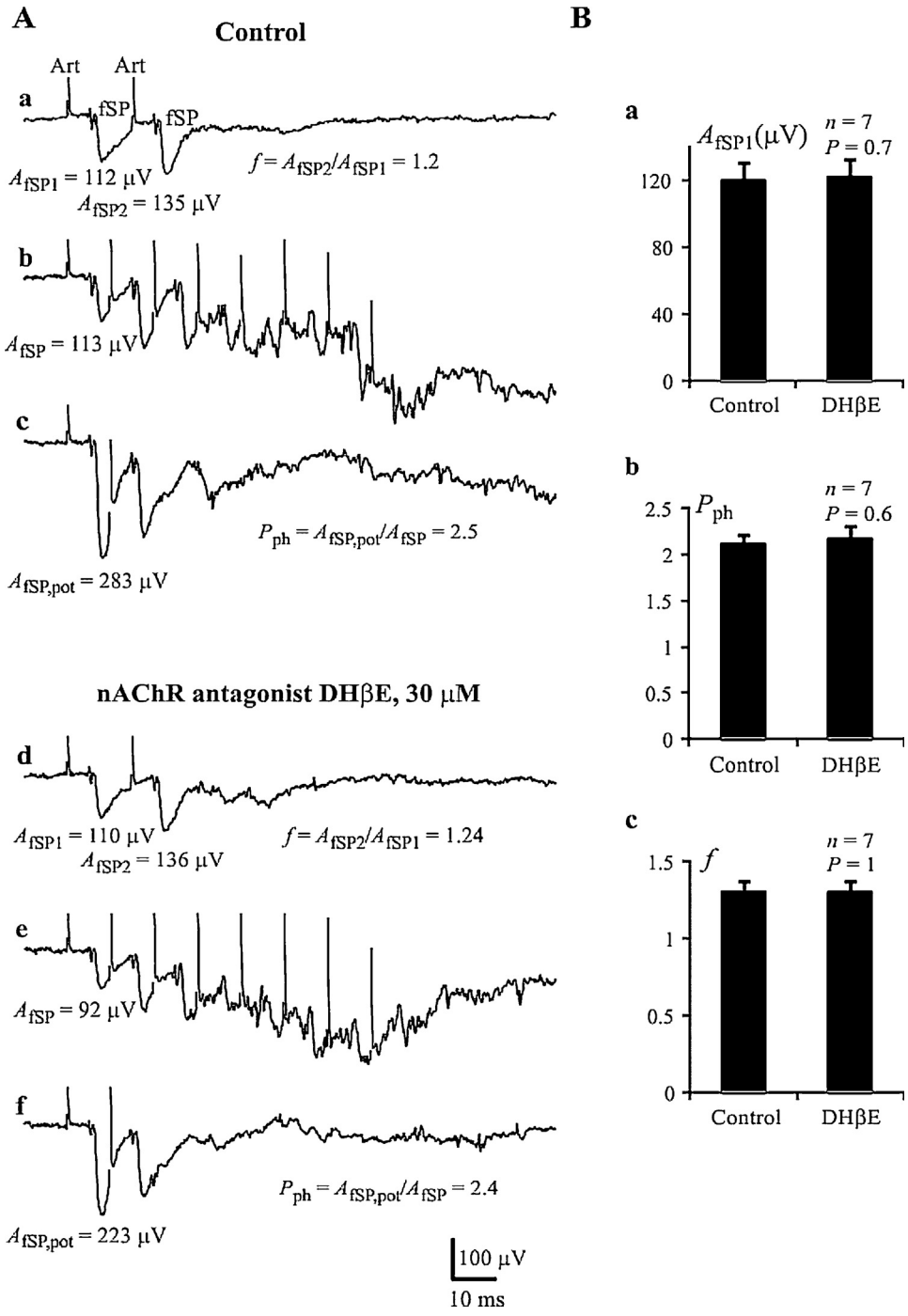

Fig. 1 – Effect of the nicotinic acetylcholine receptor antagonist, DHβE, on the retinotectal synaptic transmission. (A) Recordings from the individual experiment. (a), (d) – responses evoked in the tectum by a paired-pulse stimulation of a single retina ganglion cell at interpulse interval of 15 ms in the control conditions and when 30 μM of DHβE was present, respectively. (b), (e) – responses evoked in the tectum by a conditioning stimulation of a single retina ganglion cell with a train of 8 current pulses at interpulse intervals of 10 ms in the control conditions and when 30 μM of DHβE was present, respectively. (c), (f) – responses evoked in the tectum by a testing paired-pulse stimulation, delivered 10 s after the conditioning stimulus, in the control conditions and when 30 μM of DHβE was present. Art, stimulus artifact. fSP, individual retinotectal fast synaptic potential. A_{fSP1}, A_{fSP2}, amplitudes of the first and second fSP of the response to paired-pulse stimulus. $f = A_{fSP2}/A_{fSP1}$, paired-pulse facilitation of the retinotectal transmission. A_{fSP}, amplitude of the first fSP of the response to conditioning stimulus. $A_{fSP,pot}$, amplitude of the first fSP of the response to testing paired-pulse stimulus. The $A_{fSP,pot}$ is increased comparing to A_{fSP} due to the phasic (after-burst) potentiation of the retinotectal transmission. $P_{ph} = A_{fSP,pot}/A_{fSP}$, phasic potentiation of the retinotectal transmission. (B) Mean of 7 experiments. A_{fSP1}, amplitude of the individual fast synaptic potential of the retinotectal transmission. P_{ph}, phasic potentiation of the retinotectal transmission. f, paired-pulse facilitation of the retinotectal transmission.

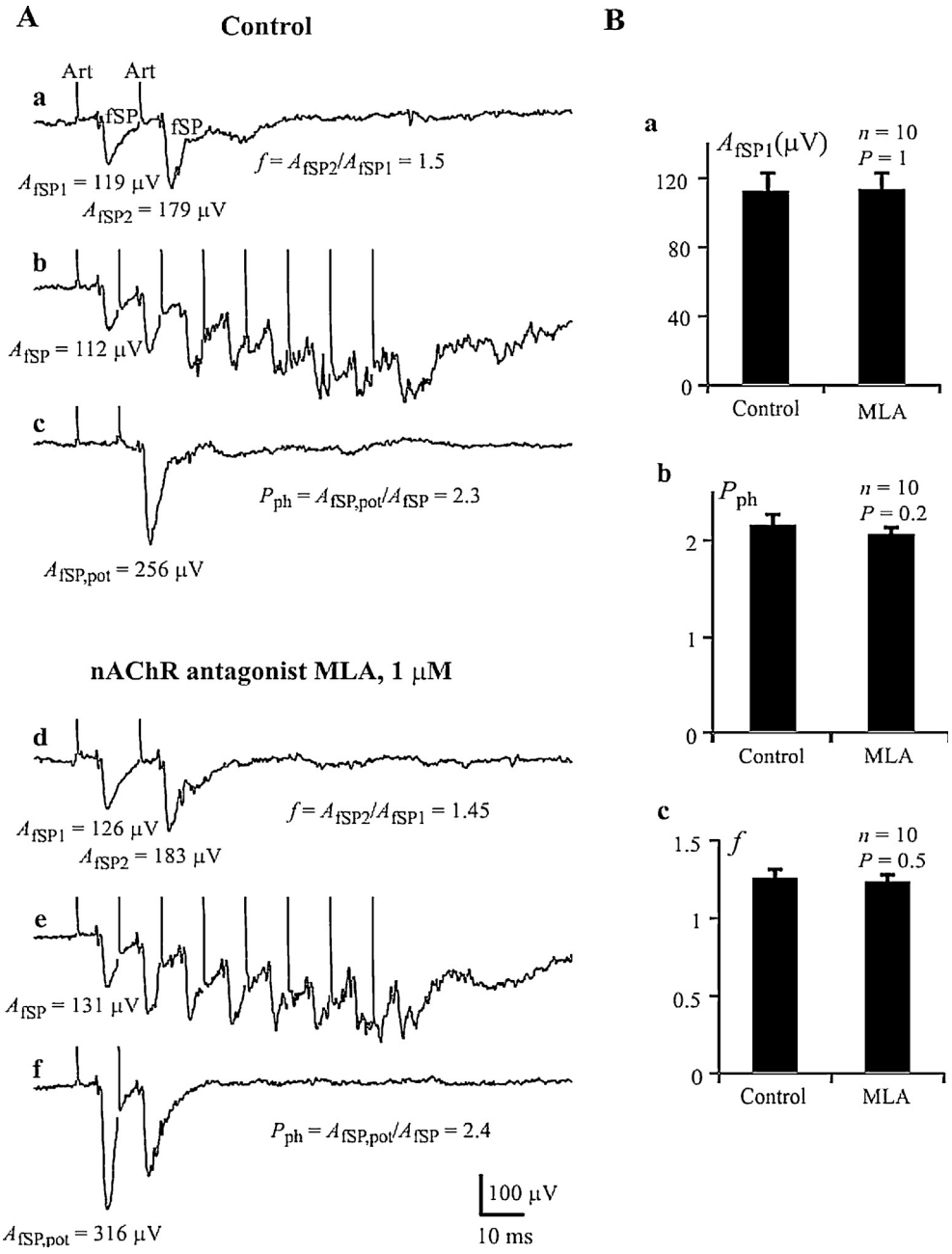

Fig. 2 – Effect of the nicotinic acetylcholine receptor antagonist, MLA, on the retinotectal synaptic transmission. For explanation of the notations see the legend of Fig. 1. Fail of the response (A(c)) to the first stimulus of the paired-pulse stimulation demonstrates unitary nature of the response.

acetylcholine receptors of the considered subtypes, the antagonists and agonists of those receptors both would lead to a decrease of the P_{ph}, approaching 1.

The results of the experiments described above have demonstrated that the nAChR antagonists MLA and DHβE, and agonists RJR-2403 (with a preference to α2β4 subtype) and choline (with a preference to α7 subtype) have had no appreciable effect on the tonic potentiation, A_{fSP1}, phasic potentiation, P_{ph}, and paired pulse facilitation, f, of the retinotectal transmission. Thus, the tonic and phasic potentiation of the frog retinotectal transmission are not mediated by the α3β2, α4β2, α2β4, α6β2 or α7 subtypes of the nicotinic acetylcholine receptors, as one could expect extrapolating the data obtained from the analysis of the presynaptic nAChR subtypes on the rat and mouse retina afferent terminals [14–16]. Application of the agonists has led to a 13% (although insignificant) increase of the amplitude of the retinotectal synaptic potential, A_{fSP1}. This could be explained by the activation of α7 and/or α2β4 receptors situated in the terminals of afferents from the nucleus isthmi. Butt et al. [6] have shown that α7-containing receptors are located on these terminals. Activation of these receptors may lead to a

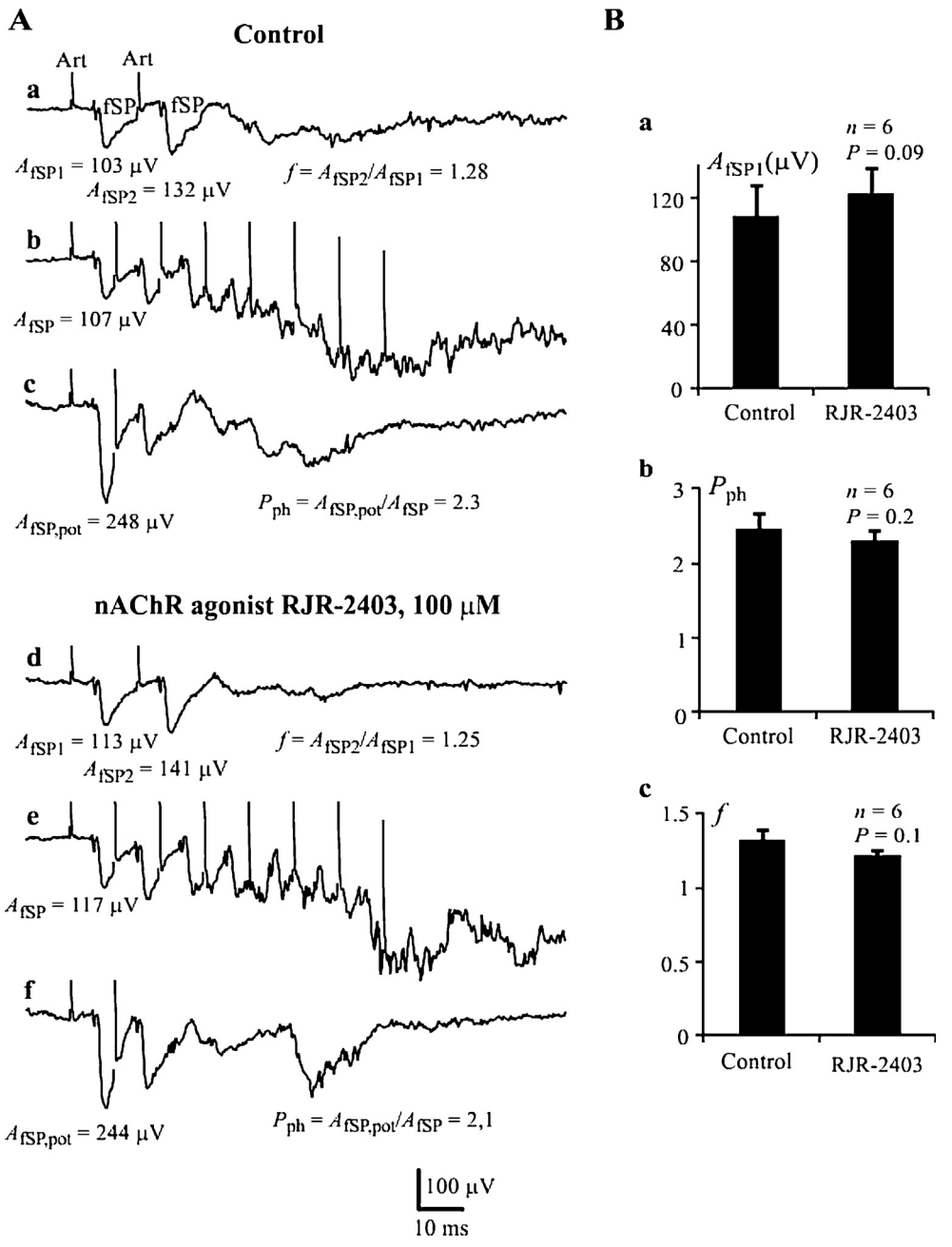

Fig. 3 – Effect of the nicotinic acetylcholine receptor agonist, RJR-2403, on the retinotectal synaptic transmission. For explanation of the notations see the legend of Fig. 1.

potentiation (auto-potentiation) of the release of the acetylcholine from these terminals. Because the ambient level of the acetylcholine in the tectum is maintained through this release, its potentiation would lead to an increased level of the ambient acetylcholine and, consequently, enhanced tonic potentiation of the retinotectal synaptic transmission (enhanced A_{fSP1}). A slight but significant decrease of the paired-pulse facilitation under the action of the choline supports this interpretation. Finally note that the nAChRs activated by agonists may undergo fast desensitization. In such a case the effect of an agonist would resemble the effect of antagonist.

A few studies on the cold-blooded animals report the activity of the considered substances. For example, Benkan and Levin [22] have demonstrated that nicotine-induced behavioral (anxiolytic) effect on the zebrafish was reversed by both MLA and DHβE. Titmus et al. [5] have found that nicotinic agonists (choline, carbachol, cytisine, nicotine) cause a reversible increase in the rate of miniature excitatory postsynaptic currents in the tectum of Xenopus frog. They also showed that the nicotinic blockers mecamylamine and MLA reduced responses to carbachol and cytisine. Papke et al. [23] have cloned the zebrafish nAChR subunits and expressed key nAChR subtypes in Xenopus

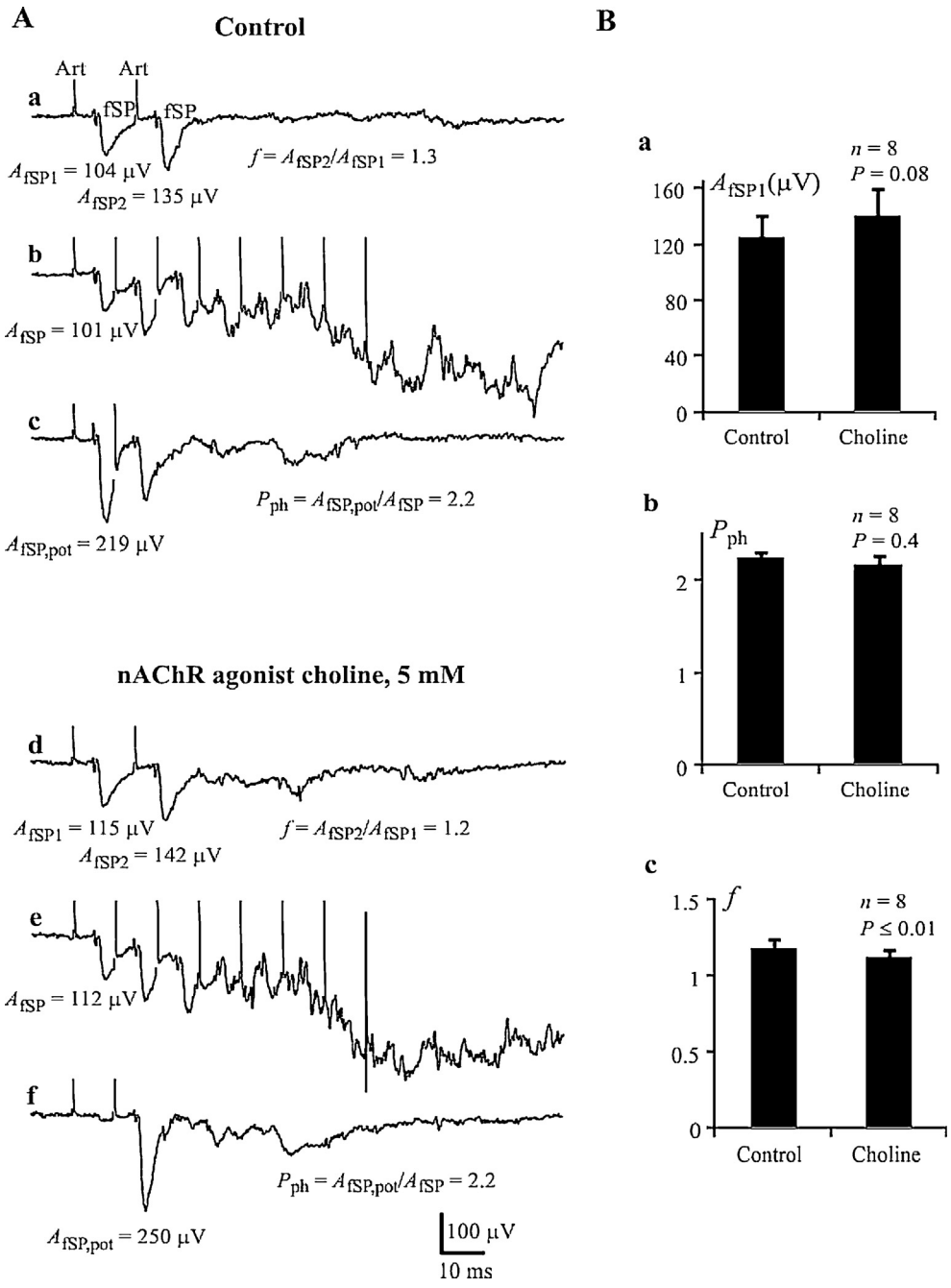

Fig. 4 – Effect of the nicotinic acetylcholine receptor agonist, choline, on the retinotectal synaptic transmission. For explanation of the notations see the legend of Fig. 1. Fail of the response (A(f)) to the first stimulus of the paired-pulse stimulation demonstrates unitary nature of the response.

frog oocytes including neuronal α4β2, α2β2, α3β4, and α7 nicotinic receptor subtypes. They showed that choline and tropane had activated both fish α7 and α4β2 nAChRs, nicotine had good potency and efficacy for fish α4β2 receptors, cytisine was full agonist for fish α7 receptors, mecamylamine was most potent for blocking fish α3β4 and β2-containing nAChR.

It was shown in our previous studies [11,12] that the presynaptic nAChRs, responsible for the tonic and phasic potentiation of the frog retinotectal transmission to the tectum layer F, are sensitive to the nicotinic acetylcholine receptor antagonist d-tubucurarine. Application of d-tubucurarine suppressed the tonic and phasic potentiation and increased the paired-pulse facilitation. Results of the present study have demonstrated that the antagonists of nAChRs of the α3β2, α4β2, α2β4, α6β2 subtypes have had no influence on the tonic and phasic potentiation and paired-pulse facilitation of the retinotectal transmission. The receptors of α3β2, α4β2, α2β4, α6β2 subtypes are found in the rat and mouse retina afferents projecting to the superior colliculus and lateral

geniculate nucleus [14–16]. So, presynaptic nAChRs situated in the terminals of frog retina afferents projecting to the F layer of the optic tectum differ from those situated in the terminals of rat and mouse retina afferents projecting to the superior colliculus (structure analogous to the frog optic tectum) and lateral geniculate nucleus. They are sensitive to nAChR antagonist d-tubucurarine but insensitive to nAChR antagonists MLA and DHβE. Probably, the subunit composition of the frog brain nicotinic acetylcholine receptors is different from the subunit composition of the mammalian brain receptors.

Possibly, frog retinotectal presynaptic nAChR are of mixed heteromeric kind, *i.e.*, they belong to the group of the nicotinic acetylcholine receptors having in their tetramer structure subunits of three different types. The mixed heteromeric nAChR have been found in the rat retina afferents [14]. It may be that the considered antagonists and agonists are not effective on the mixed heteromeric receptors. The experiment most pertinent to answer the question about the subtypes of frog retinotectal presynaptic nAChR would be the experiment with immunohistochemical labeling of the subunits of the nAChR located on the frog optic fiber terminals. Also, results of the experiments using nAChR inhibitors such as α-conotoxins (αCntx) and α-bungarotoxin (α≡gtx) may be helpful.

5. Conclusions

The presynaptic nicotinic tonic and phasic potentiation of the frog retinotectal transmission to the tectum layer F is not mediated by the α3β2, α4β2, α2β4, α6β2 or α7 nAChR subtypes. The presynaptic nAChRs situated in the terminals of the retina afferents projecting to the frog optic tectum probably are of different subtypes than the ones situated in the retina afferent terminals projecting to the mammalian superior colliculus and lateral geniculate nucleus.

Conflict of interest

The authors state no conflict of interest.

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