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Functional nicotinic acetylcholine receptors in the neurons of rat intracardiac ganglia

α-субодиничний склад нікотинових ацетилхолінових рецепторів (НХР), що експресують нейрони інтракардіальних гангліїв щура, було досліджено за допомогою моноклональних і поліклональних антитіл проти α3-, α4-, α5- та α7-субодиниць НХР. Ацетилхолінвикликані мембранні потенціали нейронів субепікардіального сплетення, що були ізольовані з лівого передсердя, вивчали методом внутрішньоклітинного відведення за наявності α-специфічних антитіл, що блокують НХР. Антитіла додавали окремо чи в різних комбінаціях. Було визначено, що нейрони інтракардіальних гангліїв експресують α3α5; α7; α7(α5) та α4-вмісні НХР. Те, що нейрони експресують різні підтипи НХР, можливо вказує на їх функціональну неоднорідність.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are the members of a gene family of ligand-gated ion channels formed by five homologous subunits oriented around a central ionic pore [11]. Neuronal nAChRs are found in the brain, where they mainly regulate the activity of dopamine and glutamate receptors, and in the autonomic ganglia, where they are involved in synaptic transmission. In contrast to muscle nAChRs, which are similar throughout the body, neuronal receptors are variable as to subunit composition and, respectively, possess various kinetic and pharmacological properties. Ganglia of different localization express different nAChR subtypes; moreover one neuron can express several nAChR subtypes; finally, neurons within one ganglion differ on the relative distribution of various nAChR subtypes [17]. The reasons for such enormous variability are not clear.

The intracardiac ganglia (ICG) belong to the parasympathetic nervous system. They are localized close to the heart atrium and their neurons project to different tissues of the heart [5, 12]. These neurons are sensitive to acetylcholine (ACh) and express the genes of several nAChR subunits [14].

Previously, by using nAChR subunit-specific antibodies in immunocytochemical experiments, we found that cultured ICG neurons express α3, α4, α5 and α7-containing nAChRs [16]. However, their functional role remained unclear. Since the antibodies generated by us were functionally active and blocked both acetylcholine-induced and synaptic currents in several ganglia of the rat and guinea-pig [7, 10, 16, 18], we employed them in electrophysiological experiments to study the contribution of each subunit-containing nAChR to the overall neuron conductivity. In the present study we describe the intracellular recordings of ACh-induced potentials in the neurons of freshly isolated ICG made in the presence of α3, α4, α5 and α7-specific antibodies applied either separately or in various combinations.

METHODS

Experiments were carried out in cardiac ganglia isolated from the subepicardium of the left

atrium and interatrial septum of the rat heart. The hearts were rapidly excised from the 21 day-old rats of either sex, decapitated under light ether anesthesia, and were mounted in a bath perfused with the Krebs solution of the following composition (mM): NaCl – 137.7; KCl – 5; $MgCl_2$ – 0.5; $CaCl_2$ – 2; KH_2PO_4 – 1; $NaHCO_3$ – 12; D-glucose – 11. The preparation of non-dissociated intracardiac ganglia was made under dissecting microscope as described earlier [15]. The preparation usually contained several small ganglia, consisting of one to tens of neurons each, and fine fiber tracts passing through the ganglia, which were observed under Nomarski differential interference optics at 400x magnification. A water immersion 40x objective was submerged into the Krebs solution that covered the preparation. Individual neurons were impaled with microelectrodes filled with 2M KCl solution (resistance 80-200 MΩ). The membrane potentials were evoked by ionophoretic application of acetylcholine (ACh) through extracellular pipette. The ionophoretic electrodes had the resistance of 50 MΩ and were filled with 1Mol/l ACh solution ('Sigma", USA). The ionophoretic currents ranged from 10 to 200 nA. The membrane potentials were amplified by standard direct current amplifier and were digitized at a frequency of 100 Hz. The results were analyzed with a self-developed computer software.

Monoclonal antibodies 1D6 and 1E10, specific, respectively, to α3 and α5 nAChR subunits, and rabbit affinity purified polyclonal antibodies (Abs) specific to α4 and α7 nAChR subunits were obtained, purified and characterized as described previously [16]. In the course of intracellular recordings they were added to perfusion solution in concentrations 30 mg/l (α3, α5 and α7-specific) and 40.5 mg/l (α4-specific). These concentrations were close to saturating ones found in our previous experiments [16, 18]. All studies were carried out at room temperature. The data obtained are presented as means ± SE.

RESULTS

In total, 27 neurons of the intracardiac ganglia were investigated. The data were obtained from the neurons exhibiting stable resting potentials throughout 15 min after punching with intracellular electrode. ACh-induced potentials exhibiting plateau after 0.5 – 1.5 s ACh-application in the absence of the Ab were used for further analysis. The amplitudes of resting membrane potentials and ACh-induced potentials varied among the neurons from -20 to -50 mV, and from 11 to 21 mV, correspondingly. The blocking effects of the Ab were hardly reversible; therefore, one neuron per preparation was usually investigated. The inhibitory effect reached plateau in 10 to 15 min after the Ab addition to perfusion solution. A depression of the ACh-potential was observed in 10 out of 18 neurons for α3-antibody (55%), in 8 out of 14 neurons for α4-antibody (57%), in 7 out of 9 neurons for α5-antibody (78%) and in 17 out of 22 neurons for α7 antibody (77%), and was equal to 22.6 ± 4.8 (n=10), 18.8 ± 4.0 (n=8), 26.1 ± 6.0 (n=7) and 25.1% ± 3.9% (n=17), correspondingly.

These data suggested that rat ICG neurons expressed nAChRs containing each of the above subunits. To clarify whether the same neuron possessed nAChRs with different α subunits, we studied the blocking effects of two or more different antibodies applied to the same neuron in succession. Each subsequent antibody was added to a perfusion solution 10 to 15 min after the application of a previous one, when the ACh-potential reached a plateau.

The combined blocking effect of two, α7- and α3-specific Abs, was observed in three neurons out of four investigated (75%) indicating for the presence of both nAChR subunits in these cells.

The combined blocking effect of three, α7-, α3- and α4-specific Abs was investigated in six neurons. The presence of α3- and α7- , as well as of α4- and α7- subunit pairs was de-

Table 1. The presence of blocking effect of three α-subunit-specific Abs in the individual neurons of the rat intracardiac ganglia

Cells	Abs specific to subunit		
	α3	α4	α7
neuron 1		+	+
neuron 2	+	+	+
neuron 3			
neuron 4	+		+
neuron 5			
neuron 6	+	+	+

tected in one neuron each. The α3-, α4- and α7- nACR subunits were present in two neurons. No blocking effect with either of the Ab tested was observed in other two neurons (Fig. a, Table 1).

In the last set of experiments we studied the combined blocking effect of four antibodies, α3-, α4-, α5- and α7-specific, applied in succession to the same neuron (Fig. b). Six neurons were analyzed in such a way. The following Ab combinations were found blocking (in one neuron each): (α3+α4+α5+α7), (α3+α4+α5), (α3+α5+α7), (α5+α7) and (α4+α7). In one neuron only α4-specific Ab exerted a blocking effect (Table 2). Each subsequent Ab increased the blocking effect of the previous one by 5 to 10%; in no case the complete depression of ACh potential was observed.

Table 2. The presence of blocking effect of four α-subunit-specific Abs in the individual neurons of the rat intracardiac ganglia

Cells	Abs specific to subunit			
	α3	α4	α5	α7
neuron 1	+	+	+	+
neuron 2	+	+	+	
neuron 3	+		+	+
neuron 4		+		+
neuron 5			+	+
neuron 6		+		

DISCUSSION

The data presented here demonstrate that the neurons of ICG express functional nAChRs containing α3-, α4-, α5- and α7-subunits that is in accord with our previous immunocytochemical data [16]. When tested with individual Abs, α3- and α4-subunits were detected in 56–57% of neurons, while α5- and α7-subunits were observed in 77–78% of neurons. These values are somewhat different from those obtained in immunocytochemical experiments, where α3-subunits was found in 32% of neurons, α4-subunit in 47% of neurons, α5-subunit in 14% of neurons and α7-subunit in 22% of ICG neurons [16]. mRNA encoding the α3 nAChR subunit was found in all ICG neurons [14]. Possibly, this difference is explained by the fact that here we worked on native ganglia, and not in cultured neurons as in two papers referred. In addition, the latter work was performed in the ganglia of newborn rats, while the properties of autonomic ganglion neurons from immature and adult animals are different [8].

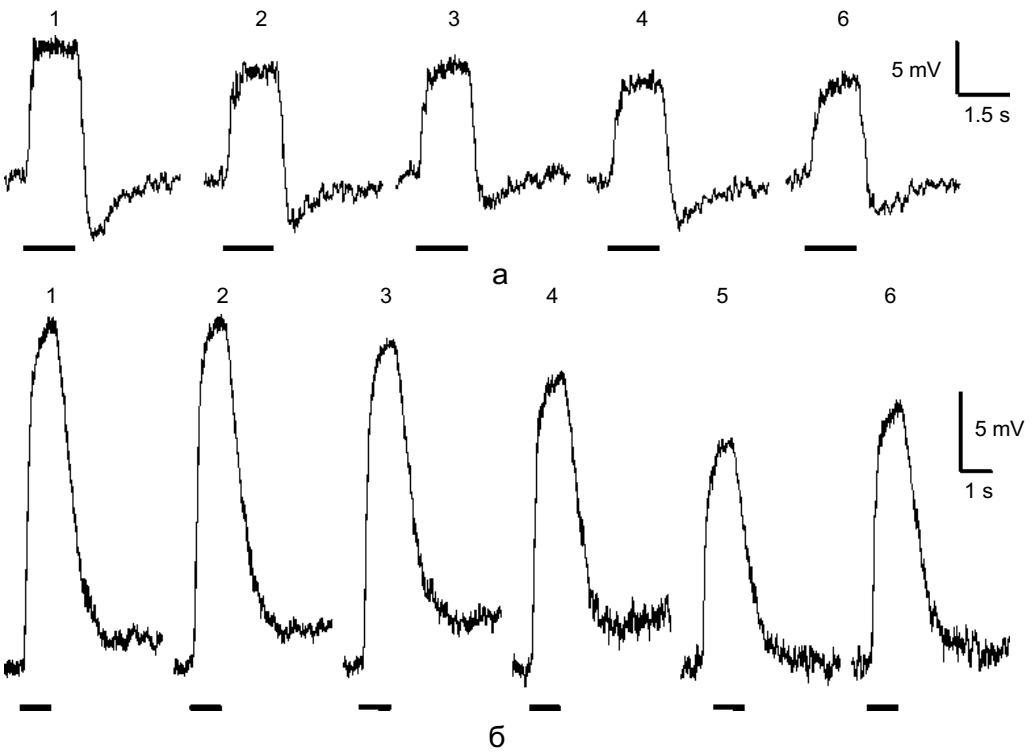
Each Ab used reduced the ACh-potential amplitude by 19 to 26%; correspondingly, the summarized depression was about 93%. According to these results, the α3-, α4-, α5- and α7-containing nAChRs, in total, were responsible for almost 100% of ACh-potential amplitude.

To detect, in which combinations nAChR subunits are found in ICG neurons, we applied two, three or four different Abs in succession. Each subsequent antibody was added when the ACh-potential reached a plateau. This approach was previously shown informative in superior cervical ganglia of the rat [18] and in the inferior mesenteric ganglia of the guinea-pig [10]. We found the additional decrease of the ACh-potential amplitude upon the application of subsequent Ab (Fig. a, b). However, the additional decrease was much less than that observed with the corresponding Ab taken alone; in no case the complete inhibition of the response was achieved. In contrast, in the rat superior cervical [18] or guinea-pig inferior mesenteric ganglia neurons [10] the same Abs applied sequentially were much more effective. From our point of view,

this may be due either to bad availability for the Ab binding of nAChRs in enzymatically non-treated ICG neurons or to close co-localization of different nAChR subtypes in the synaptic areas of these neurons. As shown by us previously in superior cervical ganglia neurons, the latter may prevent simultaneous binding of two and more Ab molecules [18]. Nevertheless, even the small additive blocking effect of certain Ab combinations indicated for the presence of corresponding nAChR subunits in the neurons studied. The results obtained demonstrate that the neurons were very heterogeneous as to the nAChR subunit combinations found, however, some generalizations can be made.

α3-subunit alone was found in 56% of neurons, in combination with either α5-or α7-subunit in 50–53% of neurons and in combination with either α4- or (α4+α7), (α5+α7) and (α4+α5) subunits in 33% of neurons (tables 1, 2). These data suggest that all α3-containing neurons also had either α7- or α5-subunit and at least half of them also had α4-subunit.

α3-subunits are important components of autonomic ganglia nAChRs [1]. As shown in canine intracardiac ganglion, the functional ganglionic transmission is mediated primarily by receptors containing α3β2 nAChRs [2]. α3-subunits are often found in combination with α5-subunits, which are also abundantly expressed in central and peripheral nervous system [3, 6, 14, 20]. We found this subunit in superior cervical and intracardiac ganglia of the rat [16], in the submucose plexus and inferior mesenteric ganglia of the guinea-pig [7, 10], as well as in the solar plexus of the guinea-pig and superior mesenteric ganglion of the mouse (Voitenko, unpublished). In the



The combined effect of three (a) and four (b) different antibodies recorded from the same neuron.
The bars under the curves indicate the time of Ach application.
Each subsequent antibody was added to a perfusion solution 10 t0 15 min after the application of previous one, when the Ach-potential reached a plateau: 1 – control, 2 – Ab α7 15 mg/ml (Fig.a), Ab α7 30 mg/ml (Fig.b), 3 – +Ab α3 30 mg/ml, 4 – +Ab α4 40,5 mg/ml, 5 – +Ab α5 30 mg/ml, 6 – wash

inferior mesenteric ganglia of the guinea-pig, α3α5-containing nAChRs were solely responsible for the synaptic transmission [10]. These data are in accord with our present results, which, therefore, suggest that ICG neurons also express α3α5-containing nAChRs. However, α5-subunits alone were found in more neurons than α3- ones (77% compared to 56%) suggesting that they might be included in the structure of other, non-α3α5 nAChRs.

α7-subunit-containing nAChRs are the second most abundant nAChR subtype found in both central and autonomic nervous system [9, 14, 17]. This is in good agreement with our data showing that about 77% of ICG neurons examined were sensitive to α7-specific Ab. According to our data, α7-containing receptors could be found on the same neurons as α3 (or α3α5)-containing ones; the same was shown previously for superior cervical ganglia [16, 18]. About a half of α7-containing neurons (33% compared to 77%) had also α5-subunit; therefore, α5-subunit could be included within heteromeric α7α5 nAChRs.

The presence of α4-containing nAChRs, which are abundant in the brain, was questionable in autonomic ganglia [4, 7, 10, 16]. But it was found in ICG [14]. Here we show that more than 50% of all neurons examined were sensitive to α4-specific Ab. This subunit was present in more than a half (33% compared to 56%) of α3-containing neurons and α7-containing neurons (41% compared to 77%). In contrast, the combination of α4+α5+α7 subunits was found in 17% of cells only (Tables 1, 2). These data allow suggesting that α4-containing nAChRs comprise a separate receptor subtype found in about a half of neurons bearing either α3(α5)- or α7-containing receptor subtypes.

Taken together, our data suggest that the neurons of the ICG of the rat express several nAChR subtypes containing the following combinations of a-subunits: α3α5; α7; α7(α5) and α4. This is in agreement with numerous literature data showing that a single neuron can express more than one receptor subtype (reviewed in [17]). The neurons studied were very heterogeneous as to the combinations of subunits expressed that is also in accord with our previous data on superior cervical ganglia neurons [16, 18]. However, the distribution of nAChR α-subunits in the neurons of intracardiac ganglia differed from that observed in the other ganglia studied with the same set of the Abs. The most pronounced difference was in the relatively high number of α4-positive neurons in ICG compared to rat superior cervical ganglia [16, 18], guinea-pig submucose [7] and solar plexuses (Voitenko, unpublished), inferior mesenteric ganglia [10] and mouse superior mesenteric ganglia (Voitenko, unpublished). The physiological significance of this difference is not clear, but it is probably due to the special functional properties of intracardiac ganglia. Cardiac parasympathetic nerves may act selectively at discrete cardiac sites possibly explaining the high heterogeneity of the ICG neurons as to their nAChR composition [13].

In conclusion, the studies presented here show that rat intracardiac ganglia neurons bear α3α5; α7; α7(α5) and α4-containing nAChRs. Each nAChR subtype is present only in a part of the neurons; the neurons are heterogeneous as to the nAChR subtypes expressed that possibly indicates their functional differences.

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FUNCTIONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN THE NEURONS OF RAT INTRACARDIAC GANGLIA

The alpha subunit composition of nicotinic acetylcholine receptors (nAChRs) expressed in the neurons of intracardiac ganglia of the rat was investigated using monoclonal and polyclonal antibodies against α3, α4, α5 and α7 nAChR subunits. The acetylcholine-induced membrane potentials in the neurons of the subepicardial plexus isolated from the left

atrium of the heart were studied by intracellular recording performed in the presence of subunit-specific nAChR-blocking antibodies applied either separately or in various combinations. It was found that intracardiac ganglia neurons express α3α5; α7; α7(α5) and α4-containing nAChRs. The neurons were heterogeneous as to the nAChR subtypes expressed that possibly indicated their functional differences.

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