Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neuroscience Letters 381 (2005) 350–353
Neuroscience Letters
www.elsevier.com/locate/neulet

Nicotinic ACh receptors in area postrema neurons of immature rat brain

Masaru Sorimachi *, Minoru Wakamori

Department of Physiology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8520, Japan

Received 12 June 2004; received in revised form 1 November 2004; accepted 21 February 2005

Abstract

The electrophysiological properties of nicotinic ACh receptors (nAChR) were investigated in acutely dissociated area postrema (AP) neurons of the immature rat brain using the whole-cell patch-clamp recording method. ACh induced a transient inward current exhibiting a strong inward rectification. The ACh response was mimicked by nicotine and cytisine, and was inhibited by nAChR antagonists, but not by 10^{-7} M atropine. Muscarinic AChR agonists did not induce any current. We confirmed the Ca^{2+} permeability of nAChR. These results indicate the presence of nAChR on AP neurons, and suggest that the activation of nAChR play important roles in cardiovascular functions in rats.

Keywords: Whole-cell patch-clamp; Area postrema neurons; ACh; Nicotinic ACh receptor; Muscarinic agonists; Atropine

The area postrema (AP) is a medullary circumventricular organ located in the hindbrain at the level of the obex, and has a dense vascular supply, which is devoid of a blood–brain barrier. In light of its location and disappearance of drug-induced emesis after ablation, the AP has long been considered a chemosensitive trigger zone for emesis (see reviews by Borison [4,5]). It has been reported that central administration of nicotinic ACh receptor (nAChR) agonists modulate cardiovascular functions in rats [9,14] and induce emesis in cats [2,3] and shrews [13] by activation of nAChR in the AP. Other lines of evidence also suggest the presence of nAChR on AP neurons. For instance, rat AP neurons express mRNA of nAChR subunits (α5)[16] and α3β4-like subtype of nAChR [12], and mouse AP neurons also express α3β4-subtype [17]. Furthermore, the excitatory effects of ACh have been demonstrated in some AP neurons of shrew brainstem slices, where ACh increased the spontaneous firing rate with extracellular unit recording [13]. However, the pre- or postsynaptic location for nAChR remained unknown in this study [13]. To investigate the postsynaptic location of nAChR, we employed single AP neurons dissociated from rat brainstem slices, and measured the nAChR agonists-induced currents using the whole-cell patch-clamp recording technique. The combined techniques of the dissociation of neurons and the patch-clamp methods allow us to clarify the electrophysiological and pharmacological properties of nAChR under the voltage-clamp conditions.

Rat AP neurons were dissociated from 14- to 20-day-old Wistar rats (Kyushu Animal Supply Co., Japan), that were decapitated under ether anesthesia. This study was approved by the Committees on Animal Experimentation of the Graduate School of Medical and Dental Sciences, Kagoshima University. The brain was quickly removed from the skull and placed in ice-cold HEPES-buffered saline containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 5.5 mM glucose. The pH of the saline solution was adjusted to 7.4 by adding tris(hydroxymethyl) aminomethane (Tris-base). The brain was sliced at a thickness of 400 μm with a microslicer (DTK-1000, Dosaka, Kyoto, Japan), and the slices were kept in bicarbonate-buffered saline bubbled continuously with 95% O_2–5% CO_2 at room temperature (21–26 °C). The bicarbonate-buffered saline contained 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 2 mM KH_2PO_4 and 5.5 mM glucose. The slices were then treated with dispase (2500 pU/ml; Goudoushusei, Japan) in bicarbonate-buffered saline for 60 min at 33 °C. The AP region, which can be easily identified from its location with a binocular microscope (Zeiss), was cut out using the tip of an injection needle. Care was taken not to involve neighbor-

* Corresponding author. Tel.: +81 99 275 5224; fax: +81 99 275 5231.
E-mail address: ciliary@m.kufm.kagoshima-u.ac.jp (M. Sorimachi).

0304-3940/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2005.02.052

ing nucleus tractus solitarius. The pieces were transferred to 35 mm culture dishes (Falcon, USA), and mechanically triturated with fire-polished glass pipettes in a range of sizes. Culture dishes were placed on the stage of an inverted microscope (TE200, Nikon, Japan), and cells were continuously superfused at a rate of 2 ml/min with saline at 24–26 °C. ACh and other drugs were rapidly applied to single cells using a Y-tube perfusion device.

Whole-cell currents were recorded using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany), and data analysis was done using Igor Pro (Wave Metrix, OR). The internal solution (patch pipette solution) contained 70 mM K-gluconate, 50 mM KCl, 10 mM NaCl, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, and 2 mM ATP-Na_2. The pH was adjusted to 7.2 with KOH. For measurement of the *I–V* relationship, Cs-based internal solution was used which contained 98 mM CsOH, 40 mM CsCl, 98 mM aspartate, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM EGTA, and 2 mM MgATP. The pH was adjusted to 7.2 with CsOH. The external solution was HEPES-buffered saline. When *N*-methyl-D-glucamine ($NMDG^+$) was totally substituted for external Na^+, or the concentration of Mg^{2+} was increased to 10 mM, the osmolarity of the solution was kept constant using an osmometer (Vogel OM801, Germany). To obtain the Ca^{2+}/Na^+ permeability ratio (P_{Ca}/P_{Na}), we measured the reversal potential (V_{rev}) of the ACh (500 μM)-induced current at a holding potential (V_H) between 0 and +30 mV with a 10 mV step in the presence of 1 or 110 mM of external Ca^{2+}. The solution of 1 or 110 mM Ca^{2+} contained 155 or 0 mM of NaCl, respectively, in addition to 10 mM HEPES (the pH was adjusted to 7.4 with NaOH or $Ca(OH)_2$, respectively). The values of P_{Ca}/P_{Cs} and P_{Na}/P_{Cs} were calculated using the formulae described by Lewis [10], assuming that the activity coefficients of Na^+, Cs^- and Ca^{2+} were 0.75, 0.75, and 0.3, respectively.

When the medium-sized AP neurons (10–12.5 μm diameter) were held at a V_H of −70 mV, the application of ACh evoked a rapidly desensitizing inward current in 833 of 1129 cells tested (74%; Fig. 1A). The smaller cells (less than 10 μm diameter) were often unresponsive to ACh. The absence of the ACh response in 26% of the medium-sized cells was not due to cell damage, because the application of GABA (50 μM) induced currents in these cells. The relationship between the peak current amplitude and ACh concentration is shown in Fig. 1B. All responses are expressed as percentages of the mean responses to the preceding and following application of 100 μM ACh, the concentration of which was optimal to minimize desensitization of the response (the second response was 99 ± 2%, $N = 13$, of the mean of preceding and following ones). The half-maximum effective concentration of ACh was 95 ± 5 μM. Application of nicotine or cytisine, nAChR agonists, also induced a current in a concentration-dependent manner (Fig. 1B). Nicotine was most effective, followed by ACh, whereas cytisine was least active. The repeated applications of higher doses of nicotine accelerated desensitization of the response, and we, therefore, employed 100 μM ACh in later experiments. Dihydro-β-erythroidine,

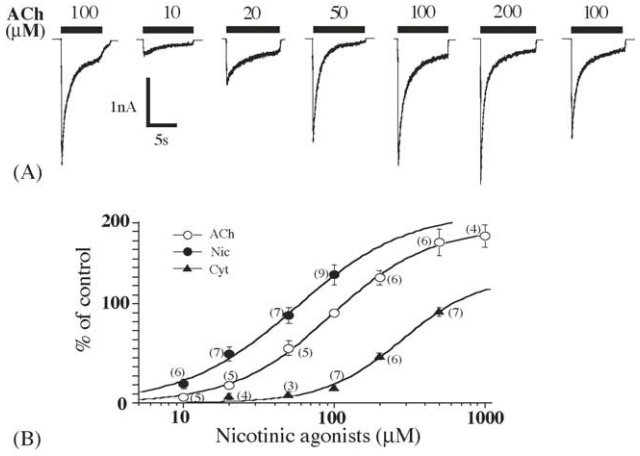

Fig. 1. ACh-induced currents in single AP neurons. (A) Representative ACh-evoked inward currents observed in a single neuron at five different ACh concentrations at a V_H of −70 mV. (B) Concentration–response relationship for the ACh-, nicotine (Nic)- and cytisine (Cyt)-induced currents. The values are expressed as percentages of the response obtained with 100 μM ACh. Each point is the mean, and the vertical bars indicate S.E.M. The number of experiments performed is shown in parentheses beside the symbols. The continuous line shows the fit of the Hill equation ($I = [\text{agonist}]^n/([\text{agonist}]^n + EC_{50}{}^n)$) to the data means.

D-tubocurarine, or mecamylamine at concentrations, which are conventionally used to inhibit nAChR, significantly inhibited the ACh response (Table 1). On the other hand, the ACh–induced current was little affected by 10^{-7} M methyllycaconitine, an α7-subtype antagonist of nAChR [1] (Table 1). This result is consistent with the finding, which showed very weak or rare signals of ^{125}I-α-bungarotoxin binding in the AP [8]. Atropine at 10^{-7} M also little affected the ACh response (Table 1), suggesting poor expression of muscarinic AChR (mAChR) on AP neurons employed. In fact, none of mAChR agonists, muscarine (200 μM, $N = 8$), oxotremorine (200 μM, $N = 7$), and pilocarpine (1 mM, $N = 7$), induced any current. Taken together, these results indicate that ACh acts

Table 1
Effects of various AChR antagonists on the ACh-induced currents

		Percentage of controls (%)
Dihydro-β-erythroidine	2×10^{-5} M	58 ± 4 (6)*
	5×10^{-5} M	20 ± 3 (9)*
D-Tubocurarine	5×10^{-5} M	20 ± 4 (10)*
Methyllycaconitine	10^{-5} M	99 ± 3 (14)
Mecamylamine	10^{-5} M	36 ± 3 (11)*
Atropine	10^{-7} M	101 ± 7% (6)

The cells were treated for 1 min with the indicated agent before stimulation with 100 μM ACh. The responses in the presence of antagonist (except for mecamylamine) were expressed as a percentage of the mean of the first and third control responses. The second ACh response in the absence of antagonist was 99 ± 2% ($N = 13$) of the mean of the first and third ACh response. The response in the presence of mecamylamine was expressed as a percentage of the preceding control, since the inhibitory effect of this agent lasted longer (the second ACh response was 91 ± 3% of the first ACh response, $N = 10$).

* Significant differences from the controls (*P* is smaller than 0.01, non-paired Student's *t*-test).