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NICOTINIC RECEPTOR SUBTYPES IN RAT SUBFORNICAL ORGAN NEURONS AND GLIAL CELLS

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Abstract—It is unclear which nicotinic acetylcholine receptor (nAChR) subtypes are involved in the nicotinic activation of cells in the subfornical organ (SFO). We investigated the nAChR subtype using molecular biological, electrophysiological, pharmacological and immunohistochemical techniques. The use of reverse transcription–polymerase chain reaction in rats demonstrated the presence of mRNAs for the α2, α3, α4, α6, α7, β2 and β4 subunits in the SFO. The characteristics and dose-dependency of nicotine-induced inward currents in many dissociated SFO neurons were similar to those induced by acetylcholine in the presence of atropine. The nicotine-induced currents were larger than those induced by cytisine in most responding cells, suggesting the predominance of the β2- rather than the β4-containing nAChR. NIC-induced currents were significantly inhibited by dihydro-β-erythroidine (a relatively selective antagonist for α4-containing nAChRs, and a partial antagonist for α2 or α3) at 300 nM in all responding cells. Additionally, the currents were significantly inhibited by α-conotoxin MII at 10 nM (a selective antagonist for α3- and/or α6-containing nAChRs) in some but not all responding cells. Methyllycaconitine at 10 nM (a selective α7-nAChR antagonist) reduced the nicotine-induced current significantly, but to a lesser extent. Fluorescence-labeled α-bungarotoxin (a homomeric α7 subtype selective binding drug) binding and immunofluorescence for the α7 subunit showed that positive images almost overlapped with those immunopositive for an astrocyte marker. These results suggest that the α4β2 subtype is the main functional receptor in SFO neurons while α2, α3, α6, and β4 subunits have some effect, and homomeric the α7 subtype exists in SFO astrocytes.

Key words: nicotine, cytisine, whole-cell recording, immunohistochemistry, RT-PCR.

The subfornical organ (SFO) situated adjacent to the third ventricle plays an important role in drinking behavior, cardiovascular regulation and vasopressin release (McKinley et al., 2001). We have demonstrated electrophysiologically that application of nicotine (NIC) to SFO slice preparations induces neural excitation (Ono et al., 2003, 2008; Inenaga et al., 2003). I.c.v. injection of NIC induces these physiological responses (Ono et al., 2008; Buccafusco and Yang, 1993; Iitake et al., 1986) and increases c-Fos immunoreactivity in the whole of the SFO (Ono et al., 2007). These studies suggest that nicotinic activation of SFO neurons is related to inductions of the physiological functions.

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels made up of combinations of a number of different nicotinic receptor subunits. They can be divided into two subfamilies, the homomeric nAChR (α7 subunit) and the heteromeric nAChR (α2–6 and β2–4 subunits) (Le Novère and Changeux, 1995). In our previous study (Ono et al., 2003), NIC-induced inward currents were inhibited by application of dihydro-β-erythroidine (DHβE), that is a relatively selective antagonist for α4-containing nAChRs. Neuron-like cells immunopositive for the anti-α4 antibody were abundant in the SFO (Ono et al., 2003), suggesting that the α4 subunit plays an important role in SFO neurons. However, since some studies have reported that DHβE is a relatively broad spectrum antagonist at non-α7 nAChRs (Harvey et al., 1996; Chavez-Noriega et al., 1997; Khiroug et al., 2004) and there are no reports of other types of nAChR subunits in the SFO, it is possible that other nAChR subunits play some roles in the nicotinic responses of SFO neurons.

In the present study, we investigated the behavior of nAChR subtypes in the SFO using molecular–biological, electrophysiological, pharmacological and immunohistochemical techniques. In our previous studies on the SFO, currents induced by bath application of NIC reached their peaks before the maximal concentration of NIC arrived at the chamber, probably due to desensitization of nAChR. Hence, in the present study, NIC was applied acutely to dissociated SFO neurons using a Y-tube system (Nakagawa et al., 1990).

EXPERIMENTAL PROCEDURES

Experimental animals

The care and handling of the animals used in these experiments were consistent with all the U.S. National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate Animal Experiment Committees of the Kyushu Dental College where the experiments were performed. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data and animal discomfort was kept to the minimum.

SFO slice preparations and acute cell dissociation

Male adult Wistar rats weighting 150–200 g were deeply anesthetized with ketamine (250 mg/kg, i.p.) and decapitated. The

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Abbreviations: ACh, acetylcholine; CTxMII, α-conotoxin MII; CYT, cytisine; DHβE, dihydro-β-erythroidine; DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein; Hex, hexamethonium chloride; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NIC, nicotine; NSE, neuron specific enolase; RT-PCR, reverse transcription–polymerase chain reaction; SFO, subfornical organ; αBgt, α-bungarotoxin.

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brain was blocked at an angle of approximately 45° between the coronal and horizontal planes from the ventral to the rostrodorsal surfaces of the brain. Slices of 300-μm thickness were prepared in a cold slice-cutting solution (in mM; sucrose 211, KCl 3, $NaHCO_3$ 26, glucose 10, $MgSO_4$ 1.3, KH_2PO_4 1.24, $CaCl_2$ 2.1). After making SFO slices, the SFO tissue was further dissected away using a dissecting microscope. As described in our previous study (Ono et al., 2005), the SFO tissues were enzymatically digested with collagenase A (1 mg/ml; Roche Diagnostics Co., Indianapolis, IN, USA), collagenase D (1 mg/ml; Roche Diagnostics Co.) and with papain (30 units/ml, Worthington Biochemical Co., Freehold, NJ, USA) at 37 °C (for 15 min in each case). After washing in culture medium (DMEM and F12 in a ratio of 1:1 in 10% fetal calf serum), they were carefully replaced in a new culture medium using a Pasteur pipette. They were mechanically dispersed with fire-polished glass pipettes (tip i.d. ranging from 200 to 500 μm). The dissociated neurons were maintained in a humidity-controlled incubator gassed with 15% O_2 and 5% CO_2 at 37 °C for 3–6 h prior to electrophysiological experimentation.

Conventional and single-cell reverse transcription–polymerase chain reaction (RT-PCR)

These procedures have described in our previous studies (Ono et al., 2005; Honda et al., 2003). After making slices, total RNA was extracted using RNeasy Mini Kits (Qiagen, Tokyo, Japan). Reverse transcription of the total RNA (40 ng) was performed in a final volume of 20 μl using oligo-dT^{12-18} primer (0.5 μg/μl) and RNasin (10 U; TaKaRa, Otsu, Japan) using the sensiscript RT kit (Qiagen). PCR was performed with a thermal cycler (PCR Thermal Cycler Device; TaKaRa). The specific primers for nAChR subunits were from published designs (Léna et al., 1999). The other specific primers for neuron specific enolase (NSE, as neuron marker) and glial fibrillary acidic protein (GFAP, as astrocyte marker) were also used from the following sequences; NSE (M11931): forward GGAGTTGGATGGGACTGAGA, reverse ACCCCCTTGAGTGTGTGGTA and GFAP (NM_017009): forward TAATGACTATCGCCGCCAAC, reverse TCCACCGTCTTTACCACGAT. These primer sets for nAChR subunits and others are designed to put intron(s) between targeted arrangement positions of primers to make it possible to discriminate amplified from genomic DNA. PCR was performed with a PCR buffer containing 10 pmol primers, 2.5 U *Taq*DNA polymerase (TaKaRa *Taq* Hot Start Version, TaKaRa Biomedicals) and each transcribed cDNA, in a final volume of 50 μl. Single-stranded cDNA products were denatured and subjected to PCR amplification (40 cycles). Each PCR cycle consisted of denaturation at 94 °C for 20 s, annealing at 58–60 °C for 30 s and finally extension at 70 °C for 35 s. The PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. Total RNA (5 ng) was used as a negative control. The dorsal root ganglion (DRG) was used as a positive control for α5 and β3 subunits.

In single-cell RT-PCR for NSE and GFAP, the entire cell contents, including the cell nucleus, were aspirated into the electrode pipette by applying negative pressure at the end of the electrophysiological recordings. Electrodes contained approximately 6 μl of sterile pipette solution (see below) to which was added RNasin (2 U) to suppress mRNA degradation during the time of recording. After aspiration, the tip of electrode was broken off, and its contents were injected into a tube containing oligo-dT^{12-18} primer (0.5 μg/μl) and RNasin (5 U) with sensiscript RT kit to give a final volume of 10 μl. Single-stranded cDNA was synthesized in an incubator at 37 °C overnight. To identify a variety of mRNAs coexpressed in single cells, a two-step protocol was used. In the first round, a single-cell cDNA sample was used as a template in a multiplex (plural pairs of primers, each 10 pmol) PCR reaction in a final volume of 100 μl, and 20 cycles were performed at 58 °C. In the second round, 2 μl of the first reaction mixture sample was used as a template with each pair of specific primers, as well as in the conventional PCR.

Whole-cell patch-clamp recordings

Dissociated cells were submerged in a dish with a volume of approximately 0.5 ml and with a perfusion flow rate of 1 ml/min at room temperature (22–24 °C). Patch pipettes were double-pulled (P-97; Sutter Instrument, Novato, CA, USA) from glass capillaries. They were adjusted to 2–5 MΩ when filled with a pipette solution (in mM): K gluconate 145, $MgCl_2$ 3, EGTA 0.2, Hepes 10 (pH 7.2 adjusted with KOH). The series resistance was compensated up to 70%. Currents were recorded using a whole-cell clamp amplifier (EPC-8; HEKA Elektronik, Lambrecht, Germany). Recorded cells were judged to be neurons if they were visually round and bright, and had a large tetrodotoxin (TTX, Wako, Osaka, Japan; at 0.3 μM) -sensitive Na^+ inward current (>3 nA) and a high input resistance (>1 GΩ) in voltage-clamp mode. Drugs were applied to cells using the Y-tube method (Nakagawa et al., 1990). The distance of Y-tube from the recorded cell was set to approximately 2 mm. When acetylcholine (ACh) chloride (Sigma, St. Louis, MO, USA) was applied to neurons, the muscarinic receptor antagonist atropine (Sigma) at 1 μM was routinely included in the perfusion solution. Such a concentration of atropine has been reported to block muscarinic responses completely (Xu et al., 2001; Honda et al., 2003). The following five drugs were used as alternative agonists and antagonists for nAChRs; cytisine (CYT), hexamethonium chloride (Hex), DHβE, methyllycaconitine (MLA) (Sigma) and α-conotoxin MII (CTxMII; Tocris, Bristol, UK). Throughout the whole of the experiments, holding membrane potential was set to −70 mV. The values of the membrane potentials were corrected for the liquid junction potentials (11 mV) present in the pipette.

FITC-labeled α-bungarotoxin (αBgt) staining for dissociated cells

For this experiment, dissociated SFO cells were centrifuged to allow them to be cultured at high density. Five hours after dissociation and culture, SFO cells were incubated with FITC-labeled αBgt (Sigma) at 1.5 μg/ml in the culture medium at 4 °C for 15 min. The dissociated cells were then fixed for 30 min in 4% paraformaldehyde in PBS. To assess non-specific binding of αBgt, the cells were preincubated in NIC at 1 mM. This decreased the intensity of FITC-fluorescence in SFO cells. The fixed cells were further processed for immunohistochemistry for GFAP. After incubating with 0.5% goat serum for 30 min, the cells were incubated with anti-GFAP (rabbit, Histofine H903, diluted 1:100, as astrocyte marker) overnight at 4°C. They were then incubated with Alexa™ 546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; diluted 1:200) for 2 h at room temperature.

Immunofluorescence for α7 subunit and GFAP

The three rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused through the left ventricle with PBS, followed by 500 ml of Zamboni's fixative (pH 7.3). The brain blocks were dissected out and immersed in the same fixative for 3 h. After cryoprotectant treatment with 10–30% sucrose in PBS, 8 μm thick coronal sections were made serially using a freezing microtome. Sections throughout the SFO were mounted on MAS-coated glass slides (Matsunami, Japan). After a 30 min incubation with 0.5% goat serum, the sections were incubated with anti-α7 subunit antibody (mouse, Sigma M-220, diluted 1:250) and anti-GFAP antibody (rabbit, Histofine H903, diluted 1:100) overnight at 4 °C. The sections were then incubated with Alexa™ 546-conjugated goat anti-mouse IgG (Molecular Probes, diluted 1:200) for the anti-α7 subunit and Alexa™ 488-conjugated goat anti-rabbit IgG (Molecular Probes, diluted 1:200) for anti-GFAP for 2 h at room temperature. For immunohistochemical negative controls, the primary antibody was omitted.