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CELLULAR AND SUBCELLULAR LOCALIZATION OF ALPHA-1 ADRENOCEPTORS IN THE RAT VISUAL CORTEX

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Abstract—Noradrenaline is thought to play modulatory roles in a number of physiological, behavioral, and cellular processes. Although many of these modulatory effects are mediated through alpha-1 adrenoceptors, basic knowledge of the cellular and subcellular distributions of these receptors is limited. We investigated the laminar distribution pattern of alpha-1 adrenoceptors in rat visual cortex, using immunohistochemistry at both light and electron microscopic levels. Affinity-purified anti-alpha-1 antibody was confirmed to react only with a single band of about 70–80 kDa in total proteins prepared from rat visual cortex. Alpha-1 adrenoceptors were widely distributed though all cortical layers, but relatively high in density in layers I, II/III, and V. Immunoreactivity was observed in both neuronal perikarya and processes including apical dendrites. In double-labeling experiments with anti-microtubule-associated protein 2, anti-neurofilament, anti-glial fibrillary acidic protein, anti-glutamic acid decarboxylase 65/67, anti-2-3-cyclic nucleotide 3-phosphodiesterase, and anti-tyrosine hydroxylase antibodies, alpha-1 adrenoceptors were found mainly in dendrites and somata of microtubule-associated protein 2-immunopositive neurons. About 20% of alpha-1 adrenoceptors were in GABAergic neurons. A small number of alpha-1 adrenoceptors were also distributed in axons of excitatory neurons, astrocytes, oligodendrocytes and noradrenergic fibers. Using an immunoelectron microscopic technique, numerous regions of alpha-1 adrenoceptor immunoreactivity were found in cell somata, on membranes of dendrites, and in postsynaptic regions. Moreover, a small number of immunoreaction products were also detected in axons and presynaptic sites.

These findings provide the first quantitative evidence regarding the cellular and subcellular localization of alpha-1 adrenoceptor immunoreactivity in visual cortex. Moreover, the ultrastructural distribution of alpha-1 adrenoceptor immunoreactivity suggests that alpha-1 adrenoceptors are transported mainly into dendrites and that they exert effects at postsynaptic sites of neurons.

Key words: noradrenaline, receptor, immunohistochemistry, immunoelectron microscopy, synapse, transport.

Biogenic amines in the brain, including those of the noradrenergic (NA) system, play important roles in cellular, physiological, and behavioral modulation. The majority of NA fibers in the brain arise from a single source of NA-containing neurons in the locus coeruleus (LC; A6) (Dahlstrom and Fuxe, 1964). NA fiber projections are widespread throughout the cerebral cortex (Ungerstedt, 1971; Maeda and Shimizu, 1972) and play important roles in the regulation of cortical function (Sara and Segal, 1991). Evoked firing patterns of neurons in the locus coeruleus are highly stereotypical; these neurons respond to a variety of afferent inputs (Nakamura, 1977; Foote et al., 1980, 1983; Aston-Jones and Bloom, 1981). The multitude of physiological effects resulting from NA activation are thus likely a result of different types of postsynaptic responses to NA released at different loci. In fact, it is possible that the various functional effects attributed to NA are the result of differential patterns of activation of adrenoceptor subtypes. NA mediates effects in the brain via several different G-protein-linked receptors, broadly classified as alpha and beta adrenoceptors (Berthelsen and Pettinger, 1977; Raymond et al., 1990).

Alpha adrenoceptors have been divided into alpha-1 and alpha-2 subtypes based on their differences in affinity for a variety of agonists and antagonists (Morrow et al., 1985; Morrow and Creese, 1986; Minneman, 1988; Han and Minneman, 1991). Ligand binding studies have demonstrated the existence of high-affinity alpha-1 adrenoceptor binding sites in the rat brain (Morrow and Creese, 1986). The distribution of alpha-1 adrenoceptors in the brain has been determined in radioligand binding studies, which have typically employed ^{3}H-prazosin or ^{125}I-2-{[b-(4-hydroxyphenyl)ethyl]aminomethyl}-1-tetralone (HEAT) as a ligand (Young and Kuhar, 1980; Jones et al., 1985).

The NA projections in visual cortex have been shown to play important roles in the formation of synapses (Blue and Parnavelas, 1982) and synaptic plasticity during develop-

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CNPase, 2-3-cyclic nucleotide 3-phosphodiesterase; DAB, 3-3-diaminobenzidine tetrahydrochloride; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; GAD65/67, glutamic acid decarboxylase 65/67; GFAP, glial fibrillary acidic protein; HEAT, 2-{[b-(4-hydroxyphenyl)ethyl]aminomethyl}-1-tetralone; IP_3, inositol 1,4,5-triphosphate; MAP2, microtubule-associated protein 2; NA, noradrenaline or noradrenergic; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.3% Triton X-100; ROI, region of interest; RT, room temperature; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; TPBS, phosphate-buffered saline containing 0.1% Tween 20; Tris–HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

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ment (Komatsu, 1996; Kirkwood et al., 1999). It has been reported that alpha-1 adrenoceptor play a major role in NA-induced intracellular Ca^{2+} concentration responses in visual cortex (Kobayashi et al., 2000). Alpha-1 adrenoceptors are required for the formation of excitatory synapses in rat visual cortex (Nakadate et al., 2006). Moreover, it has been reported that activation of alpha-1 adrenoceptors selectively suppressed the horizontal propagation of excitation in the supragranular layers of rat visual cortex (Kobayashi et al., 2000). This alpha-1 adrenoceptor-dependent suppression may play important roles in visual information processing. Full understanding of these various aspects of NA-activated alpha-1 adrenoceptor activity in visual cortex requires determination in detail of the pattern of distribution of alpha-1 adrenoceptors in the visual cortex. In the present study, using immunohistochemical and immunoelectron microscopic methods, we for the first time examined the subcellular distribution of alpha-1 adrenoceptors in rat visual cortex.

EXPERIMENTAL PROCEDURES

Animals

In this study, a total of eight Long-Evans strain male rats were used at postnatal day 56. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Research Committee of Osaka Bioscience Institute (No. 98-30). All efforts were made to minimize the suffering of animals and to reduce the number of animals used in the present study.

Alpha-1 adrenoceptor antibody

Polyclonal antibody to alpha-1 adrenoceptor (PA1-047) was purchased from Affinity BioReagents Inc. (Golden, CO, USA). It had been raised against the sequence of amino acids 339–349 of the 3rd intracellular loop of the human alpha-1 adrenoceptor sequence (FSREKKAAK). This sequence is specific to all alpha-1 adrenoceptor subtypes (alpha-1A/C, Stewart et al., 1994; 1B, Strausberg et al., 2002; 1D, Schwinn et al., 1995), being conserved in neither other adrenoceptor subtypes (alpha-2a, b; beta-1, 2, 3) nor other G-protein-coupled receptors (NCBI, BLAST program). To check the specificity of immunostaining, the antibodies were preadsorbed with synthetic peptides based on the findings of epitope mapping. Briefly, for alpha-1 adrenoceptor polyclonal antibody, 100 μg or 1 mg of synthetic peptide (FSREKKAAK) was incubated with 10 μg of antibody at 4 °C for 24 h on a rotation vortex. The supernatant solution containing blocked antibody was then tested by immunoblotting and immunohistochemical analysis, as described below.

Protein preparation for Western blot analysis

The rats were perfused through the left ventricle with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4). The brain was rapidly removed and homogenized in 10 volumes of ice-cold homogenate buffer (20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride [Tris–HCl] at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 150 mM NaCl) containing protease inhibitors (one tablet/10 ml homogenate buffer, CompleteTM Mini, Roche Diagnostics, Basel, Switzerland). Homogenates were then centrifuged at 500×*g* for 5 min at 4 °C, and the supernatants were dissolved in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 3% SDS, 5% glycerol, and 2% 2-mercaptoethanol) and then boiled for 5 min. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA) and determined with bovine serum albumin (BSA) as a reference protein as described (Bradford, 1976).

Immunoblot analysis

SDS-PAGE was performed as described (Laemmli, 1970), and Western blotting was performed using the ECL-Plus immunoblotting detection system (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions. Proteins were separated by SDS–polyacrylamide gel electrophoresis (8% gels) and electrophoretically transferred at 50 V for 60 min onto a polyvinylidene difluoride membrane (ImmobilonTM-P, Millipore Co., Bedford, MA, USA). After a blocking step of incubation with 5% (w/v) skim milk (Becton Dickinson Microbiology System, Sparks, MD, USA) and Block-Ace (Yukijirushi Nyugyo Co., Sapporo, Japan) in phosphate-buffered saline containing 0.1% Tween 20 (TPBS, pH 7.5) for 1 h at room temperature (RT), the membrane was washed and incubated with primary antibody (1.0 μg/ml in TPBS containing 1% BSA and 5% skim milk) for 1 h at RT. Then, after incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution in TPBS containing 1% BSA and 5% skim milk) for 45 min at RT, the immunoreactive bands were detected with an ECL-Plus kit (Amersham Life Science).

Tissue preparation for immunohistochemical and immunoelectron microscopic analysis

Animals were deeply anesthetized with an overdose of sodium pentobarbiturate (50 mg/kg, i.p.; Nembutal®; Abbott Laboratory, IL, USA) and perfused through the left ventricle with ice-cold 0.9% saline. For immunohistochemical and immunofluorescence analysis, fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4) was perfused. The brain was quickly removed, post-fixed overnight at 4 °C in the same fixative, cryoprotected in graded concentrations of sucrose (final 30%) in 0.1 M PB, frozen on dry ice, and cut into 50 μm coronal sections with a freezing microtome. For immunoelectron microscopic study, animals were perfused with physiological saline, followed by ice-cold fixative containing 4% paraformaldehyde, 15% saturated picric acid, and 0.1% glutaraldehyde in 0.1 M PB (pH 7.4). The brains were removed from the skulls, and then post-fixed with the same fixative for 24 h at 4°C. Brains were washed in 0.1 M PB, and then sections were cut on a microslicer (DTK-1000, Dosaka EM, Kyoto, Japan) at 50 μm thickness. Some of these sections were processed for electron microscopic analysis as described below.

Immunohistochemistry: light microscopic analysis

Immunohistochemistry was performed using the free-floating method. Fifty micrometer sections were washed in phosphate-buffered saline containing 0.3% Triton X-100 (PBS-T) and incubated at RT for 90 min in PSB-T containing 1% hydrogen peroxide. After several washes, sections were incubated with blocking solution [5% normal goat serum (Vector Laboratories, Burlingame, CA, USA), 2% BSA (Sigma Chemical Co., MO, USA), and 10% Block-Ace (Dainihon Seiyaku Co., Tokyo, Japan) in PBS-T] at RT for 2 h, and then incubated for 2 days at 4 °C with the antibody to alpha-1-adrenoceptors (2.0 μg/ml in PBS). After washes in PBS, the sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories) at RT for 2 h. The sections were then washed and reacted with avidin–biotin peroxidase complex (ABC kit, Vector Laboratories) at RT for 2 h. The sections were subsequently incubated in 50 mM Tris–HCl (pH 7.3) containing 0.05% 3-3-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) and 0.003% hydrogen peroxide. All sections were mounted on gelatin-coated slides, dehydrated through graded