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CORTICAL AND SUBCORTICAL DISTRIBUTION OF IONOTROPIC PURINERGIC RECEPTOR SUBUNIT TYPE 1 ($P2X_1R$) IMMUNOREACTIVE NEURONS IN THE RAT FOREBRAIN

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Abstract—Ionotropic purinergic receptors (P2XR) are ATP-gated cationic channels composed of seven known subunits ($P2X_{1-7}R$) and involved in different functions in neural tissue. Although their presence has been demonstrated in the brain, few studies have investigated their expression pattern. In particular, ionotropic purinergic receptor subunit type 1 ($P2X_1R$) has been observed in the cerebellum and in brainstem nuclei. The present study investigates the $P2X_1R$ expression pattern in the rat forebrain using immunohistochemistry. The specificity of the immunolabeling has been verified by Western blotting and *in situ* hybridization methods. $P2X_1R$ immunoreactivity was specifically localized in neurons, dendrites and axons throughout the forebrain. Characteristic differences in the distribution of $P2X_1R$ were observed in different cortical areas. In prefrontal, cingulate and perirhinal cortices, very intense labeling was present in neuronal bodies. In frontal, parietal, temporal and occipital cortices, immunostaining was lighter and mainly found in dendrites and axons. The hippocampal formation was intensely labeled. Labeling was present almost exclusively in dendrites and axons and never in neuronal bodies. The diencephalon was devoid of $P2X_1R$ positive neurons or fibers except for the medial habenular nucleus, which showed very intense $P2X_1R$ immunostaining. Furthermore, two subcortical regions, namely, the nucleus centralis of the amygdala and the bed nucleus of the stria terminalis, showed intense $P2X_1R$ neuronal labeling. Present data indicate that $P2X_1R$ are prevalent in forebrain areas involved in the integration of cognitive, limbic and autonomic functions. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: nucleus centralis of the amygdala, bed nucleus of stria terminalis, limbic cortex, prefrontal cortex, cingulate cortex.

ATP is a signaling molecule that acts through two classes of purinergic receptors (P2R), namely, ionotropic (P2XR) and metabotropic (P2YR) purinergic receptors. P2XR are ionotropic channels involved in fast excitatory neurotransmission, neuromodulatory actions and as sensors of tissue damage (Khakh, 2001; North, 2002; Khakh and North, 2006). Until now, seven distinct P2XR subunits ($P2XR_{1-7}$) have been cloned from mammalian species. P2XR have been identified in virtually all tissues, where their functional role has been demonstrated (Burnstock and Knight, 2004; Khakh and North, 2006). Fewer studies have investigated the distribution and function of P2XR in the CNS; however, some of these have revealed unexpectedly wide, but selective, CNS distribution (Xiang et al., 1998; Kanjhan et al., 1999; Norenberg and Illes, 2000; Yao et al., 2000). Anatomical characterization has provided some information about the expression of the different P2XR subunits. For the ionotropic purinergic receptor subunit type 2 ($P2X_2R$), which is the one most studied, a widespread pattern has been reported that includes broad sectors of the cerebral cortex, different rhinencephalic structures, many thalamic, hypothalamic and basal ganglia nuclei as well as sensory, motor and integration nuclei of the brainstem and the cerebellar cortex (Kanjhan et al., 1999). Co-localization studies have suggested an important role of the $P2X_2R$ subunit in the regulation of hypothalamic functions (Yao et al., 2003; Florenzano et al., 2006). As to the other subunits, few studies have investigated the topographical and subcellular distribution of $P2X_{1-6}R$ subunits (Loesch and Burnstock, 1998; Yao et al., 2000, 2001; Rubio and Soto, 2001). In experimental lesion models, it has been found that ionotropic purinergic receptor subunit type 1 ($P2X_1R$) and $P2X_2R$ are up-regulated in neurons after central and peripheral axotomy (Florenzano et al., 2002; Atkinson et al., 2003; Kassa et al., 2007), and a functional interactions with post-axotomy nNOS expression has been suggested (Viscomi et al., 2004; Kassa et al., 2007).

Whether or not the $P2X_1R$ subunit is present in the CNS is still a matter of debate. Indeed, $P2X_1R$ RNA transcripts were demonstrated in the cerebral cortex, hippocampus and striatum (Norenberg and Illes, 2000); $P2X_1R$ protein expression was assessed in the cerebellum

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Abbreviations: BST, bed nucleus of the stria terminalis; BSTLD, bed nucleus of the stria terminalis, lateral division, dorsal part; Ce, nucleus centralis; Cg2, posterior cingulate area; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DG, dentate gyrus; EtC, Entorhinal cortex; fr, fasciculus retroflexus; GFAP, anti-glial fibrillary acid protein; IL, infralimbic; NeuN, neuronal nuclei; PFC, prefrontal cortex; PB, phosphate buffer; PRhC, perirhinal cortex; PrL, prelimbic; P2XR, ionotropic purinergic receptors; $P2X_1R$, ionotropic purinergic receptor subunit type 1; RSGC, retrosplenial granular cortex; RT, room temperature; S, subiculum.

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and in brainstem nuclei (Loesch and Burnstock, 1998; Yao et al., 2000, 2001); and the presence of $P2X_1R$ in pyramidal neurons of the somatosensory cortex was suggested on pharmacological grounds (Pankratov et al., 2002, 2003). Recently a wide debate arose on the specificity of P2X antibodies (Ashour et al., 2006; Anderson and Nedergaard, 2006) and a multi-technical approach has been suggested to support the specificity of immunohistochemical data (Rhodes and Trimmer, 2006). The present study investigates the pattern of expression of the $P2X_1R$ subunit in the forebrain by Western blotting, immunohistochemistry and *in situ* hybridization methods.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Twelve adult male rats (Wistar, Harlan, Udine, Italy) weighing 200–250 g were used in this study. They were group-housed in standard cages and kept under a 12-h light/dark cycle in a conditioned facility. Food and water were provided *ad libitum*. All experiments were carried out in accordance with the Italian law on the use and care of laboratory animals (DL 116\92) and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The animals were transcardially perfused under deep anesthesia (Sodium pentobarbitol, 60 mg\kg, i.p.) with 150 ml of 0.9% saline at room temperature (RT) followed by 200 ml of cold 4% paraformaldehyde in a 0.1 M pH 7.4 phosphate buffer (PB). Brains were dissected, post-fixed for 2 h at RT and cryoprotected in 30% PB/sucrose at 4 °C. Then, they were frozen with dry ice and cut into 40 μm transverse, sagittal or horizontal sections with a sliding microtome. Three series of transverse sections from the frontal pole to the occipital pole were collected in PB and stored at 4 °C. The first series was Nissl stained with Cresyl Violet for anatomical reference. The second and third series were processed for immunoperoxidase detection, multiple immunofluorescence or fluorescent *in situ* hybridization. We used six animals for immunoperoxidase staining, three animals for multiple immunofluorescence *in situ* hybridization and three animals for Western blotting. All chemicals were purchased from Sigma (St. Louis, MO, USA).

Western blotting

Rats were deeply anesthetized by i.p. injections of sodium pentobarbital (60 mg/kg) and killed by decapitation. The brain was dissected, and the forebrain was taken. The tissues were homogenized, extracted with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS) in PB for 30 min at 4 °C and centrifuged for 10 min at 4 °C (14,000 r.p.m.). The supernatant was collected and sonicated, and the protein content was revealed by the Bradford method. Total protein (100 μg) from each animal was subjected to SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were saturated with 5% nonfat dried milk and incubated overnight with the primary antibody at the appropriate dilution (rabbit anti-$P2X_1R$, 1:200; mouse anti-β-actin, (Chemicon, Temecula, CA, USA) 1:2000). Membranes were then incubated with horseradish peroxidase–conjugated goat anti-rabbit (1:5000) or horse anti-mouse (1:2000) secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive bands were detected using the enhanced chemiluminescence kit (ECL, Amersham Biosciences, Little Chalfont, UK).

Histology and immunohistochemistry

Immunohistochemical procedures were performed at RT on free-floating sections. PB was used for both chemical dilution and rinses. All primary antibody solutions were prepared in PB and 0.3% Triton X-100 and incubated overnight at RT. Each incubation step was followed by three 5 min rinses in PB.

For immunoperoxidase staining, sections were treated for 5 min with 0.3% H_2O_2 to block endogenous peroxidase. They were incubated overnight in primary antiserum (polyclonal rabbit anti-$P2X_1R$, Alomone, Jerusalem, Israel) diluted 1:500, and were then incubated for 2 h in secondary antiserum, diluted 1:200 (biotinylated donkey anti-rabbit, Jackson Immunoresearch, West Grove, PA, USA). Further, sections were incubated for 1 h in avidin–biotin complex, diluted 1:100 (Vectastain elite, Vector Laboratories, Burlingame, CA, USA). As chromogen, 3,3′ diaminobenzidine 0.05% with nickel intensification was used. Finally, sections were mounted on chrome-alum–coated slides, air dried, dehydrated with ethanol, cleared in xylene and coverslipped.

Double immunofluorescence was performed using a solution of rabbit anti-P2X1R (1:100) and mouse anti-neuronal nuclei (NeuN; 1:100, (Chemicon), lot number 24010098) or rabbit anti-P2X1R receptor and mouse anti-glial fibrillary acid protein (GFAP) (1:600, Chemicon) or rabbit anti-P2X1R receptor subunits and mouse anti-OX-42 (1:200; Serotec, Kidlington, Oford, UK). Following incubation with the mix solution of primary antibodies, the sections were incubated for 2 h at RT in a solution of Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Following incubation with the mix solution of primary antibodies, the sections were incubated for 2 h at RT in a solution of Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson Immunoresearch Laboratories). Before the last rinse, sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Then they were mounted on gelatin-coated slides and coverslipped in Gel/Mount (Biomeda, Foster City, CA, USA).

Triple fluorescence was performed using sections derived by *in situ* hybridization procedures that were incubated with the anti-$P2X_1R$ (1:100) antibody. Subsequently, they were incubated for 2 h at RT in a solution of Cy3-conjugated mouse anti-digoxigenin and Cy2-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson Immunoresearch Laboratories). Before the last rinse, sections were DAPI counterstained (5 min, 1:1000; Sigma). Then they were mounted on gelatin-coated slides and coverslipped in Gel/Mount (Biomeda).

Fluorescence *in situ* hybridization

In situ hybridization experiments were performed using probes in both sense and antisense orientations. The primers used to clone the partial cDNAs from rat total brain RNA were:

$P2X_1R$ forward: 5′-GGA CAG CTC CTT TGT AGT TAT-3′

$P2X_1R$ reverse: 5′-TGG TAG ATG GGT TTG CAG TGC-3′

The cDNA was cloned into the pGemTeasy vector, linearized with *Sac*I or with *Sac*II and transcribed with T7 or Sp6 polymerases to obtain antisense or sense digoxigenin-labeled cRNA probes, respectively.

Sections were post-fixed with 4% paraformaldehyde in 0,1 M PBS for 30 min, then rinsed with 1× PBS (0.1% DEPC treated) for 5 min. Sections were permeabilized with 0.3% Triton X-100 in 1× PBS for 30 min, rinsed with 0.5× SSC (0.1% DEPC treated) for 10 min and incubated in 500 μl of pre-hybridization buffer (2× SSC, 25% formamide, 1% Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml heparin, 0.5 mg/ml *E. coli* tRNA and 0.25 mg/ml of denatured salmon sperm DNA) at 42 °C for 2–3 h. After the prehybridization, 700 ng of digoxigenin-labeled cRNA probe was added to each section. Hybridization was performed overnight at 55 °C. The next day, sections were washed twice with 2× SSC/1 mM EDTA (10 min each), treated with 002 μg/ml RNAseA for 30 min and then washed twice with 2× SSC/1 mM EDTA. The stringency wash was performed at 55 °C for 2 h in 0.5× SSC/1 mM EDTA.