P2X7 Receptor Expression after Ischemia in the Cerebral Cortex of Rats

HEIKE FRANKE, PHD, ALBRECHT GÜNTER, PHD, JENS GROSCHE, PHD, RENATE SCHMIDT, PHD, STEFFEN ROSSNER, PHD, ROBERT REINHARDT, PHD, HEIDI FABER-ZUSCHRATTER, PHD, DIETMAR SCHNEIDER, MD, AND PETER ILLES, MD

Abstract. Large amounts of adenosine 5′-triphosphate (ATP) released from cellular sources under pathological conditions such as ischemia may activate purinoceptors of the P2X and P2Y types. In the present study, the expression of the P2X7 receptor-subtype in the brain cortex of spontaneously hypertensive rats was investigated using a permanent focal cerebral ischemia model. Immunocytochemistry with antibodies raised against the intracellular C-terminus of the P2X7 receptor showed a time-dependent upregulation of labeled cells in the peri-infarct region after right middle cerebral artery occlusion (MCAO) in comparison to controls. Double immunofluorescence visualized with confocal laser scanning microscopy indicated the localization of the P2X7 receptor after ischemia on microglial cells (after 1 and 4 days), on tubulin βIII-labeled neurons (after 4 and 7 days), and on glial fibrillary acidic protein (GFAP)-positive astrocytes (after 4 days). In the following experiments, changes occurring 4 days after MCAO were investigated in detail. Western blot analysis of the cortical tissue around the area of necrosis indicated an increase in the P2X7 receptor protein. Immunoelectron microscopy revealed the receptor localization on synapses (presynaptically), on dendrites, as well as on the nuclear membrane of neurons (postsynaptically) and glial cells. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling in combination with P2X7 receptor immunocytochemistry indicated a co-expression on the apoptotic cells. Active caspase 3 was especially observed on GFAP-positive astrocytes. In conclusion, the present data demonstrate a postischemic, time-dependent upregulation of the P2X7 receptor-subtype on neurons and glial cells and suggest a role for this receptor in the pathophysiology of cerebral ischemia in vivo.

Key Words: Adenosine 5′-triphosphate (ATP); Apoptosis; Astrogliosis; Brain injury; Neurons; P2 receptors.

INTRODUCTION

Extracellular adenosine 5′-triphosphate (ATP) serves as a mediator of intercellular communication in the immune and nervous system by triggering a variety of biological responses including excitatory transmitter function, mitogenic stimulation, and induction of cell death (1–3). Currently, 7 cloned P2X receptor-subtypes (P2X1–7) mediating the short-term effect of nucleotides have been identified. Investigations at the light and electron microscopic level have revealed that in the nervous system the P2X7 receptor-subtype appears to be limited to activated microglia, lymphocytes, macrophages, or astroglia, in keeping with its role in brain repair following inflammation, infarction, or immune insult (4, 5). A limited number of studies suggested that the P2X7 receptor-subtype exhibits circumscribed neuronal expression on motor nerve terminals (6), hippocampal mossy fiber synapses (7), cortical synaptosomal terminals (8), as well as on hippocampal neurons of rats (9).

The P2X7 receptor is bifunctional. When stimulated by low concentrations of agonists, it acts as a nonselective channel permeable to small cations. After prolonged application of higher agonist concentrations, membrane pores are created which leak molecules less than 900 daltons in size (10). In many cell types, activation of the P2X7 receptor leads to rapid cytoskeletal rearrangements, such as membrane blebbing and cell lysis (11, 12). Both necrosis and apoptosis in response to ATP stimulation have been documented (13, 14).

Under pathological conditions such as hypoxia or ischemia, extracellular purine nucleotides leak from damaged cells and, thereby, may reach high concentrations in the extracellular space. This may lead to the opening of P2X7 receptor-pores (15,16). However, the understanding of the physiological and pathophysiological roles of the P2X7 receptor-subtype in the central nervous system is still rather limited. We have demonstrated that stab wound-induced astrogliosis in vivo is accompanied by an astrocytic upregulation of the P2X7 receptor-subtype (17, 18). P2X7 receptor-immunopositive cells (most likely activated microglia) were also identified in the penumbra after occlusion of the right middle cerebral artery in rats (19).

In the present study, spontaneously hypertensive rats were investigated 1, 4, and 7 days after permanent focal cerebral ischemia induced by right middle cerebral artery...
occlusion (MCAO) in order to determine the existence and possible temporal changes of the P2X, receptor-subtype on neurons, astrocytes, and microglial cells in the peri-infarct region, and to draw conclusions on the possible roles of these receptors in apoptosis and necrosis.

MATERIALS AND METHODS

Materials

The following antibodies and conjugated markers were used: rabbit anti-P2X, receptor-subtype (Lot # AN-01, AN-02; intracellular C-terminus binding; Alomone Labs, Jerusalem, Israel); mouse anti-tubulin βIII (directed against the neuronal βIII-isoform of tubulin), rabbit anti-active caspase 3 (Promega, Madison, WI); mouse anti-rat OX 42 (CD 11b; complement receptor type 3; Serotec, Oxford, UK); mouse anti-glial fibrillary acidic protein (GFAP), biotinylated Griffonia simplicifolia agglutinin isoelectric B₄ (GSA-B4; Sigma, Deisenhofen, Germany); biotinylated Protein A (Calbiochem, La Jolla, CA); Cy2-conjugated goat anti-mouse IgG, Cy2-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-rabbit IgG, Cy3-conjugated streptavidin (Jackson ImmunoResearch, Baltimore, MD); Cell death detection kit (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; TUNEL (Roche Diagnostics, Mannheim, Germany); mouse anti-tubulin (Bayer, Leverkusen, Germany); and thiopental natrium (Trapanil, WI); mouse anti-rat OX 42 (CD 11b; complement receptor type 3; Serotec, Oxford, UK); mouse anti-glial fibrillary acidic protein (GFAP), biotinylated Griffonia simplicifolia agglutinin isoelectric B₄ (GSA-B4; Sigma, Deisenhofen, Germany); biotinylated Protein A (Calbiochem, La Jolla, CA); Cy2-conjugated goat anti-mouse IgG, Cy2-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-rabbit IgG, Cy3-conjugated streptavidin (Jackson ImmunoResearch, Baltimore, MD); Cell death detection kit (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; TUNEL (Roche Diagnostics, Mannheim, Germany). In addition ketamine hydrochloride (Ketanest®; Bayer). Then, preparative surgery was done as previously described (20). The middle cerebral artery was elevated by using a steel hook maneuvered via a micromanipulator the artery was electrocauterized. Sham operated animals underwent the same procedure except cauterizing the middle cerebral artery. Untreated animals were used as controls. Animals were investigated 1, 4, and 7 days after MCAO.

Western Blot Analysis

For the Western blot analysis, 4 days after ischemia the rats were decapitated and the cortex around the infarct zone was isolated. For control, comparable cortical tissue samples were excised and immediately frozen and stored at −70°C. The frozen tissue was resuspended and homogenized in phosphate buffer (pH 7.4; 0.06 M potassium phosphate, 1 mM EDTA). Protein concentrations were measured according to the method of Bradford (21).

Aliquots of 7.5 µg protein each (as duplicates) were separated electrophoretically on 12% resolving polyacrylamide mini-gels using a Mini Protein II electrophoresis unit (Bio-Rad Laboratories, München, Germany) and then quantitatively transferred to nitrocellulose sheets (0.45 µm). After incubation for 1 hour in TRIS-buffered saline (TBS) containing 5% membrane blocking reagent, the membranes were exposed to primary rabbit anti-P2X, antibody (1:500, type 1 antibody) for 1 hour. Subsequently, the sheets were incubated with biotinylated anti-rabbit antibody (1:1,000; Amersham Pharma Biotech, Buckinghamshire, UK) for 1 hour, followed by incubation of the blots with diluted streptavidin-horseradish-peroxidase (1: 2,000; Amersham) for 20 min. Chemiluminescence (ECL) reagents and ECL hyperfilm (Amersham) were used for detection. The specificity of the P2X, receptor antibody was determined by preadsorption experiments using a control antigen (lysophilized peptide, MW 2685 Da; Alomone). After 1 hour preincubation (1 µg peptide per 1 µg antibody), the immunoblotting was performed as described above.

The content of P2X, protein was detected by enhanced chemiluminescence. The intensity of the staining was quantified using a ScanJet 4c scanner (Hewlett Packard) and the Sigma Gel software from Jandel Scientific. Staining of the preparations from control animals was taken as 100%.

Histological Staining

Four days after MCAO the rats were transcardially perfused under thiopental sodium anesthesia with paraformaldehyde (2%) in sodium acetate buffer (pH 6.5; solution A), followed by paraformaldehyde (2%)/glutaraldehyde (0.1%) in sodium borate buffer (pH 8.5; solution B). Serial coronal sections (50-µm-thick) containing the infarct area and the comparable region in the controls were obtained by using a vibratome (TSE Technical & Scientific Equipment, Homburg, Germany) and collected as free-floating slices in 0.1 M TBS (0.05 M; pH 7.6).

For histological staining, the slices were mounted onto gelatine-coated slides and stained with celestine blue (Sigma)/acid fuchsin (Fluka Chemie, Buchs, Switzerland) according to standard procedures. The stained sections were dehydrated in a series of graded ethanol, processed through n-butylacetate, embedded in entellan (Merck, Darmstadt, Germany), and coverslipped. The slices were analyzed qualitatively as previously described (22). The neurons can be characterized by their middle cerebral artery was elevated by using a steel hook maneuvered via a micromanipulator the artery was electrocauterized. Sham operated animals underwent the same procedure except cauterizing the middle cerebral artery. Untreated animals were used as controls. Animals were investigated 1, 4, and 7 days after MCAO.

different staining quality. Intact cells possessed clearly defined blue-stained, round nuclei and practically no stained cytoplasm. A sign of profound cell injury is acidophilia, visible in densely red or red-violet staining of the cytoplasm and no visible nuclei. Intermediate cells (reversibly injured) were dark blue and had oval or triangular cell bodies with still visible nuclei (23, 24).

Immunocytochemistry

The P2X<sub>1</sub> receptor-subtype was studied by applying the rabbit antibody raised against the P2X<sub>1</sub> receptor (1:600), containing 10% normal goat serum (NGS) and 0.1% Triton X-100 for 48 hours at 4°C, followed by incubation with goat biotinylated anti-rabbit immunoglobulin (1:65; Vector Labs, Burlingame, CA) for 2 hours at room temperature. For the detection of the P2X<sub>1</sub> receptor antibody, the streptavidin/biotin technique (1:125, StreptABComplex; DAKO, Glostrup, Denmark) and the 3,3'-diaminobenzidine tetrahydrochloride (0.05%, DAB; Sigma) reaction were used. GFAP and OX 42-DAB labeling were performed using biotinylated Protein A (1:400).

Double Immunofluorescence

The P2X<sub>1</sub> immunofluorescence on neurons and astrocytes was determined in the following manner. After washing with TBS and blocking with 5% fetal calf serum (FCS) in TBS, the slices were incubated in an antibody mixture of the rabbit P2X<sub>1</sub> receptor antibody (1:600; type 1 and 2, respectively) and the neuronal marker mouse anti-tubulin βIII (1:400), or the marker of fibrous astrocytes mouse anti-GFAP (1:1,000), with 0.1% Triton X-100, 5% FCS in TBS for 48 hours at 4°C. The secondary antibodies employed to visualize the colocalization of the 2 primary antibodies were Cy3-conjugated goat anti-rabbit IgG (1:1,000) and Cy2-conjugated goat anti-mouse IgG (1:400), respectively. The sections were washed 3 times for 5 min each in 5% FCS in TBS and then incubated for 2 hours in a solution containing the mixture of the secondary antibodies with 5% FCS in TBS at room temperature.

The P2X<sub>1</sub> immunofluorescence on microglial cells was determined by incubating the sections with the P2X<sub>1</sub> receptor antibody, and subsequent detection with Cy3-conjugated goat anti-rabbit IgG (1:1,000), followed by incubation with GSA-B4 (1:200) and Cy2-conjugated streptavidin (1:350). When using mouse anti-OX 42 (CD11b; 1:100; another microglial marker), Cy2-conjugated goat anti-mouse IgG (1:400) was employed for the detection.

The active caspase 3 immunofluorescence was performed by blocking the slices as described above and incubating them in an antibody mixture of rabbit anti-active caspase 3 (1:500) and mouse anti-tubulin βIII (1:400), mouse anti-GFAP (1:1,000), or mouse anti-OX 42 (1:100) with 0.1% Triton X-100 in 5% FCS in TBS for 24 hours at 4°C. As secondary antibodies, Cy3-conjugated goat anti-rabbit IgG (1:1,000) and Cy2-conjugated goat anti-mouse IgG (1:400) were used. The incubation of the slices with rabbit anti-active caspase 3 (1:500) and GSA-B4 (1:200) was followed by treatment with Cy2-conjugated goat anti-rabbit IgG (1:400) and Cy3-conjugated streptavidin (1:200). Control experiments were performed without the primary P2X<sub>1</sub> receptor antibody or by preadsorption of the antibody with the immunizing peptides.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the in situ cell death detection kit (Roche Diagnostics GmbH) according to the manufacturer’s protocol. Briefly, brain sections were mounted onto gelatine-coated slides, permeabilized with a solution of 0.1% Triton X-100 in phosphate buffered saline (PBS, pH 7.4), and washed with PBS. Free 3'-OH termini of DNA, which are generated by DNase during apoptotic cell death, were labeled by incorporation of fluorescein-dUTP, employing terminal transferase. The TUNEL procedure was followed by immunocytochemistry using the antibodies against P2X<sub>1</sub>, or active caspase 3 in combination with Cy3-conjugated goat anti-rabbit IgG. After intensive washing, the stained sections were dehydrated in a series of graded ethanol, processed through n-butylacetate, covered with entellan (Merck) and analyzed by confocal laser scanning microscopy.

Confocal Microscopy

The double immunofluorescence was investigated by a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) to reveal the colocalization of the antibodies on neurons, astrocytes, or microglial cells. In every case the 2 reaction products were distinguished by their different fluorescence. The argon laser emitting at 488 nm was used for the Cy2-labeled antigens (yellow-green immunofluorescence) and a helium/neon laser emitting at 543 nm for the Cy3-labeled antigens (red immunofluorescence).

Electron Microscopy

For electron microscopy, control animals (MCAO rats 4 days after ischemia) were transcardially perfused (as described previously) by using 1% glutaraldehyde in solution B. Seventy-μm-thick slices were prepared and used for immunocytochemistry with the P2X<sub>1</sub> receptor antibody without permeabilization with Triton X-100. For detection, the streptavidin/biotin technique (1:125, StreptABComplex; DAKO) and DAB (0.05%) reaction were used. Following the DAB-reaction, the tissue sections were rinsed with buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 hour. They were dehydrated in 70% ethanol for 10 min and then block-contrasted in 1% uranyl acetate in 70% ethanol for 1 hour. This was followed by dehydration in 80%, 90%, 96%, and 100% ethanol for 10 min each.

Finally, the sections were flat embedded in Durcupan ACM (Fluka) between foils and the resin was polymerized at 70°C for 2 days. The region of interest was dissected with a scalpel under stereo-microscopic guidance and glued onto blank blocks of resin. Ultrathin sections (50–70 nm) were cut on an Ultracut S ultramicrotome (Reichert, Depew, NY) and collected on Formvar-coated slot grids of copper. They were stained with uranyl acetate and lead citrate and examined in a LEO 906 E electron microscope (LEO Electron Microscopy Group, Oberkochen, Germany).

RESULTS

After permanent middle cerebral artery occlusion, the ischemic damage caused a topographically demarcated zone on the infarcted hemisphere of the rat brain. The center of the ischemic lesion (core region, 4 days after...
ischemia, for example see Fig. 1Aa) was located in the frontal and sensorimotor regions of the cortex and to a minor extent in the cortex of the occipital lobe. This localization was highly reproducible as shown previously by Brint et al (25).

Light Microscopic Observation of Cellular Damage

In the present study, the altered receptor expression was investigated in cortical regions in the peri-infarct area, as demonstrated in Figure 1Ab. Changes in cell
Fig. 2. Confocal images of double immunofluorescence to characterize the localization of the P2X7 receptor subtype (red Cy3 immunofluorescence) on microglial cells labeled by GSA-B4 (yellow-green Cy2 immunofluorescence) in the peri-infarct area 1 day (A–F), 4 days (G–I), and 7 days (J–L) after MCAO. A–C: P2X7 receptor-expression on GSA-B4-positive microglial cells situated at the peri-infarct area. D–F: No double labeling on activated microglial cell at the immediate border of the infarct area. G–I: P2X7 receptor labeling on a low number of microglial cells (thin arrow) and on endothelial cells (thick arrow) after 4 days. J–L: No double labeling after 7 days. Scale bars: A–F, J–L = 20 μm; G–I = 50 μm.
Fig. 3. Confocal images of double immunofluorescence to characterize the localization of the P2X$_7$ receptor-subtype (red Cy3 immunofluorescence) on neurons, astrocytes, or microglial cells (yellow-green Cy2 immunofluorescence) in the peri-infarct area 4 days after MCAO. A–E: Localization of the P2X$_7$ receptor-subtype on neurons and (F–I, arrow) or astrocytes after ischemia. D, F: Clear intracellular labeling of the P2X$_7$ receptor-subtype on the nuclear membrane (D, thick arrow) and on the plasma membrane (D, thin arrow) in greater magnification. E: Example of the localization of the receptor on neuronal terminals (arrow). Scale bars: A–C, E = 50 μm; F–I = 20 μm; D = 10 μm.
morphology after ischemia in this region are clearly discernible in celestine blue/acid fuchsin-stained horizontal brain sections (Fig. 1Ba). In the majority of the investigated MCAO-rats, a higher number of darkly basophilic neurons (reversibly injured) than clearly red-stained neurons (profoundly injured) was observed, as shown in greater magnification in Figure 1Ba. Many of the acidophilic, red-stained and condensed dark basophilic neurons were found in the cortical layer II/III. The severity of the damage decreased with the distance to the core region (animals used, n = 3).

Light Microscopic Immunocytochemistry of P2X7 Receptors as well as Astrocytic and Microglial Markers

Immunocytochemical investigations were performed on brain slices from MCAO, sham operated, and untreated rats (3–6 animals used per group and time point). Using the antibody raised against the intracellular C-terminus of the P2X7 receptor-subtype, a time-dependent upregulation and distribution of P2X7 receptor-labeling in the region around the area of necrosis was found in comparison to the controls (Fig. 1C–F). The core region lacked P2X7-positive cellular processes. There was no detectable expression of the P2X7 receptor in controls and sham operated animals (Fig. 1C), in spite of the slight expression of the respective protein in the Western blot (see below). One day after ischemia, process-bearing cells around the infarct area were labeled for P2X7 receptors (Fig. 1D, arrows). Four days after ischemia, single P2X7 receptor-labeled cells were observed adjacent to the infarct area, e.g. in the corpus callosum, striatum, and dorsal hippocampus. At the same time, upregulation of P2X7 labeling was found in the immediate neighborhood of the infarct area. The antibody labeled the cytoplasm and nuclear membrane of the cells (Fig. 1E, arrow); additionally, P2X7-positive cellular processes were observed (Fig. 1E, arrowhead). After a postischemic period of 7 days, only a few neuronal cell bodies, in contrast to a great number of fibers, were labeled (Fig. 1F).

When slices were incubated with PBS instead of the primary antibody, or with primary antibody serum that had been preabsorbed with peptide antigen for 1 hour before use, no immunofluorescence with either of the control procedures was observed.

The GFAP-positive astrocytes in the cortical regions of controls showed GFAP-immunoreactivity of constant density. In sham operated animals, slightly increased GFAP immunoreactivity compared to untreated controls was observed. In MCAO animals, astrogliosis clearly increased from day 1 to day 4, whereas at day 7 the astrogliotic process was less pronounced. In all cases, a marked astrocytic reaction, characterized by an increase in GFAP immunoreactivity, cell hypertrophy, and proliferation was found around the infarct area participating in the formation of a gliotic scar. The progressive enhancement of GFAP expression propagated from the peri-infarct region into the hemisphere. Elevation of GFAP immunoreactivity was observed in the adjacent areas such as the corpus callosum, striatum, and hippocampus. Activated astrocytes were also found in the contralateral hemisphere of the MCAO rats. However, GFAP immunoreactivity was completely absent within the infarct zone.

The microglial cells were detected by immunostaining with the monoclonal antibody OX-42 (CD 11b) or using the microglia/macrophage marker GSA-B4 (isolectin). Both immunohistochemistry and lectin histochemistry showed similar signs of microglial activation. OX 42/GSA-B4-immunolabeling was observed at the plasma membrane and in the cytoplasm of microglial cells. Resting microglial cells, especially observed in the control animals and farther from the infarct area, were faintly stained, had small somata, and long, thin processes with several thin side branches. The activation was accompanied by a morphological transition with loss of the typical ramified morphology; intense immunostaining was observed, the somata of the cells were swollen, and the cellular processes became shorter and thicker. One day after ischemia, resting microglial cells and a low number of activated cells were found. After 4 days, strong microglial activation was observed in the peri-infarct region, which increased further after 7 days in total. Round and amoeboid cells became predominant, surrounding the infarct zone. Whereas astrocytes were located in a ring-like fashion around the ischemic core, activated microglial cells infiltrated the peri-infarct region, as well as more distant areas such as the corpus callosum.

Double Immunofluorescence Study Using P2X7 Receptor Antibodies and Neuronal, Astroglial, and Microglial Markers

To show which cells express the P2X7 protein, brain sections from control, sham operated, and MCAO animals were processed for the presence of the P2X7 receptor-subtype in combination with the neuronal marker tubulin βIII, the glial marker GFAP (a specific marker of fibrous astrocytes), and the microglial markers OX 42 and GSA-B4 (3–6 animals used per time point and group).

One day after MCAO, P2X7 receptor labeling on GSA-B4-positive microglial cells was found. Double labeling was observed only on resting microglial cells (Fig. 2A–C). On round and amoeboid cells near the infarct area no double labeling was found (Fig. 2D–F). However, 4 days after ischemia there was very low but detectable co-expression of the P2X7 receptor-subtype with OX42- or GSA-B4 positivity on microglial cells in the peri-infarct region (Fig. 2G–I, thin arrow). Endothelial cells in the injured regions also appeared P2X7-positive (Fig. 2G–I, thick arrow). At a later time point (i.e.
7 days after MCAO), no P2X7 receptor expression on microglial cells was seen (Fig. 2J–L). Ependymal cells were not P2X7 receptor-immunopositive in any of the groups studied.

The neuronal marker used is directed against the neuronal βIII isoform of tubulin. Labeled cell bodies and fibers of neurons were found in the intact cortical region in control and sham operated animals. Although tubulin βIII-positive cells were observed to the border of the ischemic area (Fig. 3A), the labeling completely disappeared in the core region of the infarct. The double labeling technique indicated that the P2X7 immunoreactivity was colocalized with tubulin βIII-positive neurons in the peri-infarct area 4 days (Fig. 3A–C, D) and 7 days after ischemia, but not in untreated or sham operated rats or 1 day after ischemia. Four days after MCAO, the pyramidal layer II/III neurons were more intensely stained than those in the layers V and VI. The P2X7 immunoreactivity was visible in the cytoplasm and on the cellular processes (Fig. 3B, D, E). Furthermore, an intense immunofluorescence was observed on the nuclear membrane of the neuronal cells (Fig. 3D, thick arrow). The increase in P2X7 receptor immunoreactivity was visible in the entire peri-infarct cortex but not in the nonlesioned hemisphere. After a postischemic time of 7 days, only a low number of labeled cell bodies was found, whereas the fiber labeling appeared more intensive (Fig. 1F). Hence, the present data demonstrate that P2X7 receptors are time-dependently upregulated on neuronal cells in vivo, as a consequence of the focal ischemic event.

Using antibodies against both GFAP and the P2X7 receptor, no double labeling was observed in untreated or sham operated rats or 1 day after ischemia. Four days after MCAO, a clear colocalization of the P2X7 receptor subtype on astrocytes was found in the peri-infarct area (Fig. 3F, G–I arrow). The P2X7 receptor immunoreactivity was equally present on cell bodies and processes of the labeled astrocytes.

Both type 1 and type 2 P2X7 receptor antibodies (see Materials and Methods) were tested. It is noteworthy that all results obtained with the 2 antibodies from different sources yielded similar results for the receptor localization and expression on neurons, astrocytes, and microglial cells in the 6 animals used (results not shown).

Western Blotting of P2X7 Receptors

Western blotting of proteins isolated from the rat cortex (n = 3) 4 days after MCAO indicated that the P2X7 receptor antibody recognized a major band at an approximate molecular weight of 70 kDa (Fig. 4). This corresponds to the expected molecular weight of the P2X7 receptor as shown in other studies (6, 7). In comparison to the untreated controls (n = 3) with a faint bound (lane 1), a clear upregulation of the protein (up to 5-fold, densitometric analysis) 4 days after MCAO (Fig. 4, lane 2) was found. The preadsorption of the antisera with the control peptide abolished the staining, indicating the specificity of the P2X7 antibody used (Fig. 4, lane 3).

Electron Microscopic Localization of P2X7 Receptors

Immunoelectron microscopy, using the type 1 antibody directed against the C-terminus of the receptors, revealed no P2X7-labeling in slices of control animals in any of the structures investigated (Fig. 5A, B). There was neither labeling of the plasma membrane, the cytoplasm, the nucleus (Fig. 5A) nor of the dendrites and synapses (Fig. 5B) in the cells investigated. Four days after ischemia, the localization of the P2X7 receptor was observed in neurons and glial cells in high density adjacent to the nuclear membrane (Fig. 5C–F). An interesting finding is the intranuclear accumulation of the DAB reaction product (Fig. 5C, D), either as bands (thin arrows) or as clusters (thick arrows). Positive P2X7 labeling was also found in numerous synaptic structures (Fig. 5E, thin arrows) and in many dendrites (Fig. 5E, thick arrow). Gliial cells showed a strong P2X7 immunolabeling in the nucleus as well (Fig. 5F).
Fig. 5. Immunoelectron microscopy using the P2X7 receptor antibody directed against the C-terminus. The P2X7 receptor-subtype is identified by a DAB reaction product. A, B: Electron microscopy reveals no P2X7-labeling in neurons (A) and dendrites as well as synapses (B) of the cerebral cortex in untreated control rats. C–F: Intense reaction for the P2X7 receptor antibody on neurons and glial cells after ischemia. C, D: Localization of the reaction product on the nuclear membrane and within the nucleus, as bands (thin arrow) or as clusters (thick arrows) of the neurons investigated. E: After ischemia, presynaptic labeling (s, thin
Double Immunofluorescence Study Using P2X7 Receptor Antibodies, Neuronal, Astroglial, and Microglial Markers as well as Markers of Apoptosis

Four days after MCAO the expression of active caspase 3 as an early apoptotic marker was detected in the peri-infarct area and to a lower extent in the adjacent regions. No staining occurred in the center of the infarct area. Double labeling studies clearly revealed the presence of active caspase 3 on GFAP-positive astrocytes in the glial barrier around the necrotic area (Fig. 6A–C). Active caspase 3 was found to be localized within the nucleus of the cells. Colocalization of active caspase 3 with tubulin βIII, OX 42, or GSA-B4 was observed in a very low number of cells only (3 animals used, examples not shown).

Up to 4 days after ischemic injury, TUNEL-positive cells appeared in a high number predominantly in the infarct area and its edge. Many TUNEL-immunoreactive cells displayed fragmentation of their nuclei, indicating apoptosis. To determine whether the P2X7 receptor subtype is expressed on these apoptotic cells, the slices were processed for the presence of the P2X7 receptor in combination with TUNEL. Colocalization occurred on cells in the peri-infarct area and not in areas remote from the infarct in 3 animals (Fig. 6G, H). The P2X7 receptor positivity was found on the membrane (Fig. 6H, thick arrow), in the cytoplasm, and partially on the processes (Fig. 6G, thin arrows) of the studied cells around the nuclear DNA fragmentation (Fig. 6G,H, asterisks). A uniform colocalization of TUNEL and active caspase 3 was not observed (n = 3, not shown).

DISCUSSION

Immediately after MCAO, the cerebral blood flow dramatically decreases in the affected brain territory. The flow reduction is most pronounced in the ischemic core and leads to rapid energy failure, ATP depletion, and ionic imbalance (26). Neurons die acutely by necrosis and in a delayed fashion by apoptosis. Whereas neuronal death occurs in the ischemic core, cells in the peri-infarct area may remain viable for prolonged periods of time (27). The astrocytes in this area become reactive and start the formation of the glial scar. Resting microglia, once activated within hours, transform into phagocytic amoeboid microglia during the first postischemic days. After injury, ATP is released into the extracellular space from various cell types, nerve terminals, activated immune cells, or damaged cells (15). Additionally, following permanent MCAO, upregulation of the capacity of enzyme chains for hydrolysis of extracellular ATP and other nucleotide 5’-triphosphates was found (16).

In the present study, immunocytochemistry, Western blot analysis, and electron microscopy indicated a time-dependent upregulation of P2X7 receptors after MCAO on neurons, astrocytes, and microglial cells.

Neurons and P2X7 Receptors

The neuronal marker tubulin βIII labeled the cytoplasm and fibers of cortical cells in control and MCAO rats. The present double labeling procedure, when visualized with confocal laser scanning microscopy, clearly indicated the neuronal localization of P2X7 receptors in the peri-infarct region of ischemic but not control rats. In cultured cerebellar granule neurons, under hypoglycemic conditions for 30 to 60 min, upregulation of the P2X7 receptor-expression by about 2-fold has been described (28). These findings, including apoptotic and necrotic features of degeneration in these culture systems, support the involvement of P2X7 receptors in ATP-evoked cell death (28, 29).

Recent functional data indicate that P2X7 immunoreactivity is concentrated in presynaptic terminals in the medulla oblongata and spinal cord (6), in excitatory nerve terminals of CA1, CA3, and dentate gyrus of the hippocampus (30), in hippocampal mossy fiber synapses (7), and cortical synaptosomal terminals (8). Furthermore, the presence of the P2X7 receptor-subtype was described on neuronal cell bodies in the medulla oblongata (6) and hippocampus (9). In the present study, ischemic upregulation of P2X7 receptors was found on the presynaptic appositions of synapses, on nerve terminals, and in the cell bodies of cortical neurons 4 days after MCAO. Electron microscopy revealed that the P2X7 receptor was concentrated on the nuclear membrane, showing an intranuclear punctative DAB reaction product (9). The intracellular localization of P2X7 receptors needs additional explanation, since only receptors at the plasma membrane are able to modify the neuronal excitability. However, the regulation of a Ca2+-permeable cationic channel, located at the nuclear envelope, by cytoplasmic ATP may be a mechanism by which cellular activity can be coupled to changes in gene expression (9).

Glia and P2X7 Receptors

The activation of astrocytes, characterized by hypertrophy, proliferation, and increased GFAP immunoreactivity, described in the present study after ischemia, has also been reported earlier (31, 32). The significance of these
characteristics is still not completely elucidated, although a role in nerve cell recovery and neuronal regeneration has been suggested (33, 34). The extracellular accumulation of large amounts of purines after CNS injury may trigger reparative events via P2 receptor stimulation by promoting astrocytic proliferation (1, 35). However, the purines may not be entirely beneficial. Activation of oxidase pathways, contributing to development of neuronal damage by generating radical oxygen species, or causing dysfunction in homeostasis were also found (36).

The presence of P2X7 receptors on astrocytes has been demonstrated in cultured cells (37, 38) and in the
hippocampus of rats (39). The present double immunofluorescence study revealed no P2X receptors expression on GFAP-positive astrocytes under control conditions, but a pronounced upregulation of P2X, GFAP-labeled astrocytes 4 days after MCAO. An upregulation of P2X receptors in Müller glial cells during proliferative vitreoretinopathy also indicates the participation of this receptor-type in processes leading to the proliferation of gliotic Müller cells (40). These observations agree with our previous findings after a stab wound injury in the nucleus accumbens of rats and suggest the involvement of endogenous ATP in the process of astrogliosis (35, 41) via activation of the P2X receptors (17, 18).

Collo et al have previously reported similar findings, but suggested that the upregulation of P2X receptors immunoreactivity is solely assigned to the activated microglia in the brain tissue surrounding the necrotic region (19). We also found a pronounced, but not exclusive P2X receptor expression on OX 42 (CD11b)- or GSA-B4-positive microglial cells after 24 hours, which decreased 4 days after MCAO. Thereby, activation of P2X receptors may contribute to inflammatory responses mediated by microglia cells (4).

Apoptosis and P2X, Receptors

Apoptosis, or programmed cell death, is important in acute and chronic pathological processes. P2X receptors are known to be involved in apoptosis (13, 14). Contributing mechanisms and markers of cell death are the activation of caspase 3 during the early stages of the execution phase of apoptosis (42), and eventually DNA fragmentation visualized in tissue sections by positive TUNEL reaction (27).

The presence of caspase 3 on astrocytes in the peri-infarct region 4 days after MCAO was demonstrated in the present study, whereas only a few caspase 3-labeled neurons and microglial cells were found at the same time point. The observed nuclear localization of caspase 3 correlates with its well-established role as an inactivator of nuclear protein and with previous studies demonstrating the same localization in cells undergoing apoptosis (43). Furthermore, Caspase 3-mediated neuronal and glial cell degeneration has been found in different experimental models of cerebral ischemia both in vitro (44) and in vivo (43, 45). Finally, using an ecto-P2X receptor antibody (recognizing an extracellular domain of the rat P2X receptor), colocalization with active caspase 3 was found (results in preparation). Whereas active caspase 3 indicates a dynamic degenerative process, TUNEL marks the end stage of severe irreversible cell damage (46). This explains the present finding that TUNEL-marked cells in the peri-infarct area were not labeled for active caspase 3. Davoli et al described such an association between caspase 3 and ischemic cell death following transient MCAO (47). In the present experiments, dual staining of TUNEL with the P2X receptor antibody confirmed the presence of this receptor on the apoptotic cells in the peri-infarct area 4 days after MCAO.

Pathophysiological Significance

This is the first immunocytochemical demonstration of P2X receptor expression by neurons and glial cells in the peri-infarct region at different time points after permanent MCAO. ATP release from dying cells could play a trophic role or indicate cell injury. Cell death in ischemia is a prolonged process that involves apoptotic mechanisms probably mediated via upregulated P2X receptors. In fact, the wide-range P2 receptor antagonist suramin, which also has NMDA receptor antagonistic properties (48), reduced the infarct volume in a rat MCAO study (49). This finding may indicate that interference with the ATPergic (or glutamatergic) excitatory system could provide neuroprotection from brain ischemia. However, a recent study documented that transient MCAO with reperfusion both in wild-type and P2X receptor-deleted mice caused similar infarct volumes when evaluated 24 hours after MCAO (50). In the present experiments, all immunohistochemical evaluations were performed 4 days after MCAO, a time point when apoptotic changes may become evident.

A recent finding of our group may provide an additional explanation for this discrepancy by showing that upregulation of P2X immunoreactivity was observed in GABAergic cerebrocortical cells kept in a culture system after transient hypoxic incubation in a glucose-free medium (51). The lack of glucose and oxygen has been found to greatly increase responses mediated by presynaptic P2X receptors (release of GABA), but not to alter responses mediated by postsynaptic P2X receptors (inward membrane current and increase of [Ca^{2+}]). Hence, in the brain, ischemia may upregulate P2X receptors of opposing functions. Whereas an augmentation of excitatory ATPergic and glutamatergic systems may facilitate the ischemia-induced neuronal damage, the augmentation of GABAergic systems may be neuroprotective. Depending on the specific conditions of a given study (e.g. time of evaluation after delivering the ischemic stimulus) P2X receptors may increase, decrease, or even fail to alter the infarct size. Another possibility is that the P2X receptors detected by our immunohistochemical methods are not responding to their endogenous agonist ATP. A number of polymorphisms in the P2X receptor gene have been observed that yield nonfunctional receptor protein (52, 53) and further point mutations may also cause trafficking defects or impaired function (54, 55). Since, in human lymphocytes and monocytes, enhanced trafficking of intracellular P2X receptors to the cell surface has been
suggested to occur during pore formation (56), it is possible that MCAO causes a P2X gene defect and subsequently promotes increased expression of nonfunctional receptors in the neuronal membrane.

The present results unequivocally indicate a time- and cell type-specific expression of P2X receptors after permanent MCAO. In addition to the postulated involvement of the P2X receptor in apoptosis, the expression of this receptor in neurons (processes and cell bodies) suggests an involvement in neuronal transmission analogous to that discussed for the monkey retina (57). It will require further experiments to determine whether MCAO-induced upregulation of P2X receptors, immunoreactivity reflects functionally intact or impaired receptors and whether neurotransmission mediated by certain neurons (glutamatergic, GABAergic) is preferentially altered.

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REFERENCES

48. Peoples RW, Li C. Inhibition of NMDA-gated ion channels by the P2 purinoceptor antagonist suramin and reactive blue 2 in mouse hippocampal neurons. Br J Pharmacol 1998;124:400–408

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