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Expression of P2X Receptors on Rat Microglial Cells During Early Development

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KEY WORDS
microglial cells; P2X receptors; development; immunocytochemistry, RT-PCR

ABSTRACT
We have used single- and double-labeling immunfluorescence and reverse transcription-polymerase chain reaction (RT-PCR) methods to examine expression of P2X receptor subtypes on microglial cells of brain in late embryonic and postnatal rat, in the N9 microglial cell line and primary cultured microglial cells. $P2X_1$, $P2X_4$, and $P2X_7$ receptors were shown on microglial cells from late embryonic day 16. Almost all the microglial cells that were positive for the marker ED1, expressed $P2X_1$ and $P2X_4$ receptors, whereas only about 30% of the cells with ED1-immunoreactivity were found to express the $P2X_7$ receptor. Positive cells were localized mainly in the white matter and around ventricles. From postnatal day 7, many microglial cells with $P2X_4$ receptor-immunoreactivity were seen around the blood vessels. At postnatal day 30, microglial cells with $P2X_1$ receptor-immunoreactivity disappeared and the cells with $P2X_4$ receptor-immunoreactivity were mainly localized around blood vessels and lining the subarachnoid space. From postnatal day 30, the microglial cells with $P2X_7$ receptor-immunoreactivity were found to be distributed widely in the forebrain. Cells with $P2X_7$ receptor-immunoreactivity from P30 were not labeled by ED1, but some were labeled by isolectin B4. The expression of $P2X_1$, $P2X_4$, and $P2X_7$ receptor mRNA and protein on primary cultures of rat microglial cells and on the N9 microglial cell line was demonstrated with immunocytochemistry and RT-PCR. This is the first report that the $P2X_1$ receptor is expressed on microglial cells, at least in early development, before postnatal day 30.

INTRODUCTION

Microglial cells are the immune effector cells that participate in many different diseases in the central nervous system (Gehrmann et al., 1995; Kreutzberg, 1996). ATP is a major factor mediating intercellular communication in the immune and nervous systems and triggers a variety of strikingly different biological effects (Dubyak and El-Moatassim, 1993; Apasov et al., 1995; Burnstock, 2001). P2 purinoceptors mediate ATP signal transduction (Burnstock, 1997; Dunn et al., 2001) and are currently classified into two subtypes, P2X and P2Y, based on molecular and pharmacological properties (Burnstock and Kennedy, 1985; Abbracchio and Burnstock, 1994). Seven different members of the P2X family of ligand-gated ion channels have been cloned (Ralevic and Burnstock, 1998; North, 2002).

Previous studies showed that microglial cells express P2X receptors and expression of P2X purinoceptors in primary cultured microglia cells or microglial cell lines focused mainly on the $P2X_7$ subunit (Kettenmann et al., 1993; Ferrari et al., 1996; Chessell et al., 1997; Collo et al., 1997; Visentin et al., 1999; Moller et al., 2000; Verderio and Matteoli, 2001; Chakfe et al., 2002; Kaya et al., 2002), although recently the $P2X_4$ receptor subunit was demonstrated on activated microglial cells in vivo (Cavaliere et al., 2003; Tsuda et al., 2003) and $P2Y_{12}$ receptors have also been described (Sasaki et al., 2003). Little is known about the expression levels of P2X receptors during the late embryonic and early postnatal periods when immigration of microglial precursors into the developing CNS occurs (Perry et al., 1985; Ashwell, 1991; Milligan et al., 1991; Navascués et al., 1996; Dalmau et al., 1998).

In the present study, we used antibodies against the seven P2X receptor subtypes to study the expression of microglial cells of rat brain during early development and compared microglial cells in different conditions, including microglial cells in vivo, primary cultures of microglial cells and microglial cell lines, using immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR) methods.

MATERIALS AND METHODS

Animals and Tissue Preparation

Breeding, maintenance, and killing of the animals used in this study followed the principles of good laboratory animal care and experimentation in compliance with Home Office (UK) regulations covering Schedule One Procedures and in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. All protocols were approved by the local animal ethics com-

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mittee. Wistar rats at the prenatal stage of development (embryonic day [E]16, E18, E20) and postnatal stages (postnatal day [P]1, P7, P14, P30, P60) were used. The rats were killed by asphyxiation with CO_2 and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. The forebrains were removed and refixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, overnight. The forebrain blocks were then transferred to 20% sucrose in phosphate-buffered saline (PBS) and kept in the solution until they sank to the bottom. Thereafter, the forebrain blocks were rapidly frozen by immersion in isopentane at −70°C for 2 min. Sections of 20-μm thickness were cut in a cryostat and floated in PBS.

Cell Culture

Microglia were obtained from primary cell cultures of cerebral hemispheres of neonatal rats (P1 to P3 rats) as described previously (Nakajima et al., 1989, 1992).

After 2–3 weeks in primary mixed cell culture, microglia were prepared as floating cell suspensions by shaking growth flasks on an orbital shaker (80 rpm, 5 min). For the immunostaining study, aliquots of the cell suspension (2–4×10^4 cells) were transferred to wells of an 8-well chamber slide (Nunc, Naperville, IL) and allowed to adhere at 37°C. After 15 min, unattached cells were removed by rinsing twice with serum-free Dulbecco's modified Eagle's medium (DMEM). After the final rinse, 0.3 ml of DMEM with 10% heat-inactivated fetal bovine serum (FBS), 2 mM of glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin was added to each well, and the slides were kept at 5% CO_2 at 37°C for 24 h. For RT-PCR analysis, the cell suspension obtained from the mixed culture was seeded on plastic dishes and allowed to adhere for 30 min at 37°C. Unattached cells were then removed after shaking the dishes gently for 5 min. The purity of microglia was almost 100%. The N9 murine microglial cell line was a kind gift from Dr. P. Ricciardi-Castagnoli (Università Degli Studi di Milano-Bicocca, Milan, Italy). These cells have been extensively used as representatives of mouse microglial cells (Ferrari et al., 1996, 1997; Cui et al., 2002; Zhang et al., 2003). The cells were grown in DMEM supplemented with 5% heat-inactivated FBS, 2 mM of glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μm of 2-mercaptoethanol. Cells were used 24 h after replating. The cells were fixed with 4% paraformaldehyde prepared in PBS, for 30 min and then used for immunostaining. For RT-PCR, the cells were collected from the flasks in cold PBS.

Abbreviations

CNS	central nervous system
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
E16	embryonic day16
FITC	fluorescein-5-isothiocyanate
HRP	horseradish peroxidase
IB4	isolectin B4
PBS	phosphate-buffered saline
RT-PCR	reverse transcription polymerase chain reaction
TRITC	tetramethylrhodamine isothiocyanate

Immunohistochemistry

The development and specificity of the P2X polyclonal antibodies has been reported previously (Xiang et al., 1998; Oglesby et al., 1999). This immunostaining protocol was performed for the floating sections of developing rat forebrains. The sections were washed 3 × 5 min in 0.01 mol/L pH 7.2 PBS, then incubated in 1.0% H_2O_2 for 30 min to block the endogenous peroxidase. The sections were preincubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ antibodies, diluted 1:1,000 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS), overnight at 4°C. Subsequently the sections were incubated with biotinylated donkey-anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:500 in antibody dilution solution for 1 h at room temperature, and then with streptavidin-horseradish peroxidase (HRP; Sigma Chemical Co., Poole, UK) diluted 1:1,000 in PBS for 1 h at room temperature. Finally, P2X immunoreactivity was visualized using the fluorescein-conjugated tyramide amplification system (NEL701, Perkin-Elmer, Berks, UK). All the incubations and reactions were separated by 3 × 5 min washes in PBS. After P2X immunostaining, the sections were incubated with the second set of primary antibodies: ED1(mouse-anti-rat; Chemicon, Harrow, UK) diluted 1:600, biotinylated isolectin B4 (Sigma) diluted 1:200 in antiserum dilution solution, overnight at 4°C. Subsequently the sections were incubated with Cy3-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch) diluted 1:300 or TRITC-streptavidin (Amersham plc, Little Chalfont, UK) diluted 1:200 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3 × 10 min washes in PBS.

The following immunostaining protocol was performed for cell cultures: cell specimens were washed 3 × 5 min in PBS, then pre-incubated in antibody dilution solution for 30 min, followed by incubation with $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ antibodies, diluted 1:400 overnight at 4°C. Subsequently, the preparations were incubated with Cy3-conjugated donkey-anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:300 for P2X antibodies and FITC-conjugated isolectin B4 (Sigma) diluted 1:200 for microglial marker, in antibody dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3× 10 min washes in PBS. In order to calculate the purity of microglial cells, counterstaining with 4′-b-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei. Control experiments were carried out with $P2X_1$–$P2X_7$ antisera absorbed with the relevant cognate peptide.

RT-PCR

Total RNA was extracted from primary cultured microglial cells and the N9 microglial cell line using the SV-Total RNA Isolation System (Promega, Southampton, UK). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham). Reverse transcription was performed

using the Moloney murine leukemia virus reverse transcriptase. Primer sequences for $P2X_{1-7}$ (Shibuya et al., 1999) were used for amplification reactions, as reported previously. Reverse transcription and cDNA amplification for all the P2X receptors was carried out with a thermal cycler (Hybaid, Ashford, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham). Every sample was further treated with Amplification Grade DNase I (Sigma) to remove any residual DNA present that could generate false-positive results. Briefly, 1 μg of total RNA was reverse-transcribed using the pd(T)12–18 as the first-strand primer at 42°C for 30 min and the enzyme was denatured at 95°C for 5 min. The sequence specific primers were then added to the reaction mixture, and the following PCR cycling parameters were used: 95°C for 45 s, 62°C for 1 min (58°C for $P2X_1$, 60°C for $P2X_2$, $P2X_3$, $P2X_5$, and $P2X_6$, 62°C for $P2X_4$ and $P2X_7$), 72°C for 1 min for 35 cycles, followed by a further stage of 10-min extension at 72°C. The resulting PCR products were resolved in a 2% agarose gel containing ethidium bromide and observed under ultraviolet (UV) illumination. RT-PCR results for all tissues were confirmed by repetition with at least three separate RNA samples, prepared from primary cultured and N9 microglial cells. Control experiments were conducted by denaturing the reverse transcriptase (95°C for 10 min) before the RT-PCR reaction.

RESULTS

Expression of P2X Receptors in the Developing Rat Forebrain

At embryonic stage E16, P2X receptors were expressed on microglial cells. Among seven members of the P2X family, only three of them ($P2X_1$, $P2X_4$, and $P2X_7$ receptors) were expressed, although the expression level and the number of positive cells differed. $P2X_1$ and $P2X_4$ receptor-immunoreactivity (-ir) was similar and the number of microglia with $P2X_1$ and $P2X_4$ receptor-ir was greater than those with $P2X_7$ receptor-ir at this stage. Almost all the microglial cells with $P2X_1$ and $P2X_4$ receptor-ir expressed ED1-ir and vice versa, almost all the cells with ED1 expressed $P2X_1$ and $P2X_4$ receptor-ir. However, only some (about 30%) of the cells with ED1-ir were found to stain positively for the $P2X_7$ receptor (Fig. 1A–C). Positive cells were mainly localized in white matter, around the ventricles. Most of them were round or oval in shape and the positive signal was seen in the cytoplasm. Some of them, especially those with $P2X_4$-ir had a few short branches. At E18, E20, P1, and P7, the distribution pattern of the cells with $P2X_1$-ir, $P2X_4$-ir and $P2X_7$-ir was almost the same as that at E16.

At postnatal stage P14, most of the cells with $P2X_4$ and $P2X_7$ receptor-ir had a few branches, although the distribution pattern was similar to that at earlier stages (Fig. 2A,C,D). Many cells with $P2X_4$ receptor-ir were demonstrated around blood vessels and these cells also expressed ED1 (Fig. 2B), but no cells with $P2X_1$ and $P2X_7$ receptor-ir were demonstrated around the blood vessels at any of the stages studied. At P30, the cells with $P2X_1$ receptor-ir disappeared and the distribution pattern of the cells with $P2X_4$ receptor-ir changed. No cells with $P2X_4$ receptor-ir were demonstrated in the white matter or in the areas around ventricles. Cells with $P2X_4$ receptor-ir were mainly localized around the blood vessels and subarachnoid spaces (Fig. 2E). The distribution pattern of the cells with $P2X_7$ receptor-ir was different from that with $P2X_4$ receptor-ir. Those cells with ED1-ir around blood vessels did not express $P2X_7$ receptor-ir (Fig. 2E), while the cells with $P2X_7$ receptor-ir were found to be widely distributed in the forebrain. At this stage, the cells with $P2X_7$ receptor-ir were not labeled by ED1 (Fig. 2E), but some were labeled by IB4 (Fig. 2F). At P60 the results were similar to those at P30.

Expression of P2X Receptors on Primary Cultured Microglial Cells and the N9 Microglial Cell Line

The immunoreactivities of $P2X_1$, $P2X_4$, and $P2X_7$ receptors were demonstrated on the primary cultured microglial cells. The distribution patterns of the positive signals on those cells showed some differences between the three receptors. The positive signals for the $P2X_4$ receptor were located mainly in the cell body around, but not within the nuclei. For $P2X_1$ and $P2X_7$ receptors, the positive signals were located in the cell body around the nuclei and also in the processes of the microglial cells (Fig. 3A–C); 98% of primary cultured cells were labeled by FITC-conjugated isolectin B4 (Fig. 2G–I), which means the purity of microglial cells was about 98%. The immunoreactivities of $P2X_1$, $P2X_4$, and $P2X_7$ receptors were also demonstrated on the N9 microglial cell line, but the distribution patterns of positive signals were not different (Fig. 3D–F).

Immunostaining Controls

No staining was observed in the preparations incubated with antibody solutions pre-absorbed with $P2X_1$, $P2X_4$, and $P2X_7$ subtype peptides.

Expression of P2X Receptor mRNA in the Primary Cultured Microglial Cells and N9 Microglial Cell Line

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of primary cultured microglial cells showed that clear bands were detected for $P2X_1$, $P2X_4$, and $P2X_7$ receptor mRNAs. No detectable bands were found for $P2X_2$, $P2X_3$, $P2X_5$, and $P2X_6$. The results from N9 microglial cell line were the same as that from primary cultured microglial cells (Fig. 4A,B). The expected size of $P2X_1$, $P2X_4$, and $P2X_7$ cDNAs is 452, 447, and 354 bp, respectively. The size of cDNA bands for $P2X_1$,

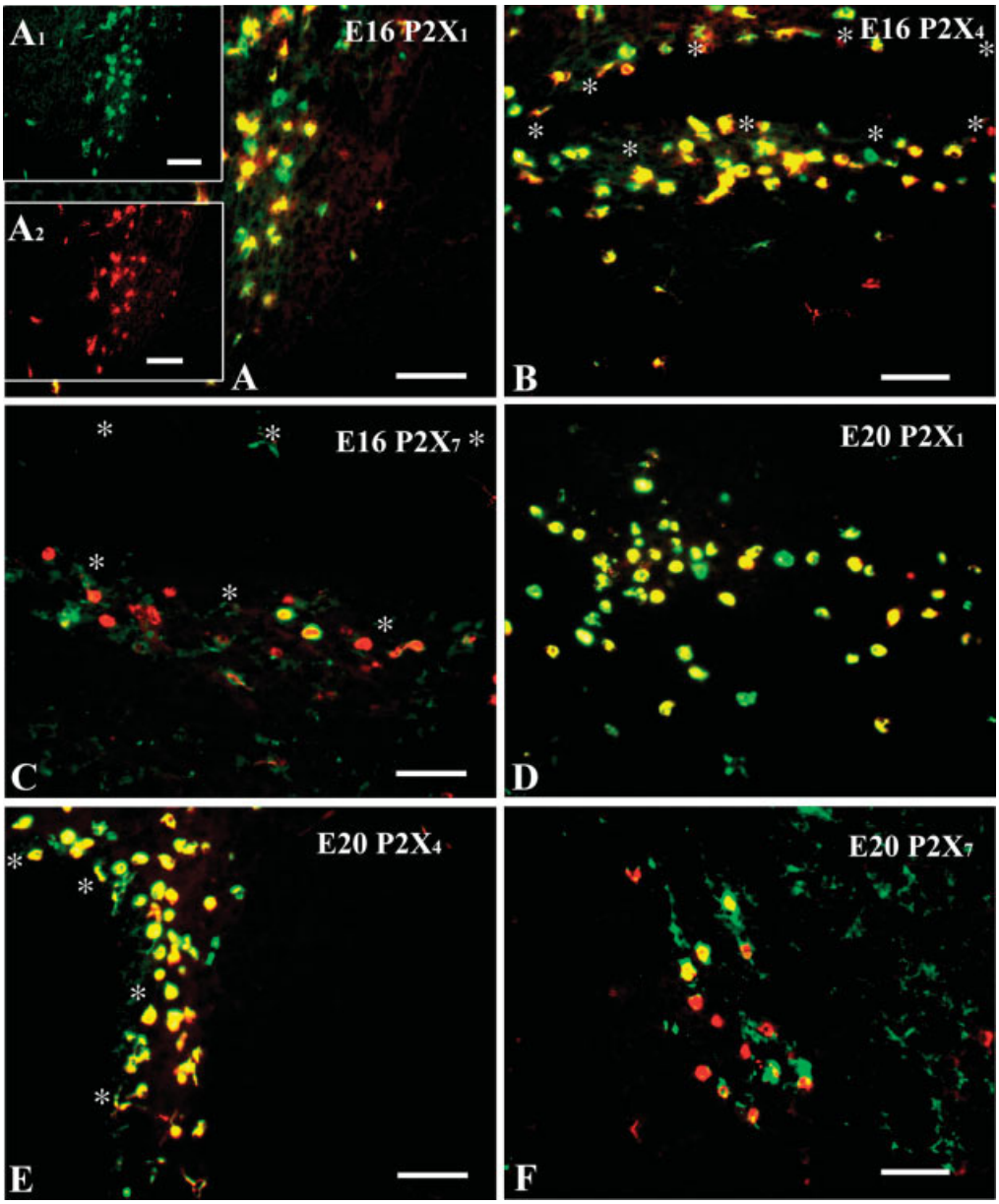

Fig. 1. Coexpression of P2X receptors and ED1 on the microglial cells in the forebrain from E16 to 20. P2X receptor immunostaining is shown in green, ED1-immunostaining is red and colocalization of P2X receptors and ED1 is in yellow. **A:** Coexpression of the $P2X_1$ receptor and microglial cell marker (ED1) in the corpus callosum at E16. **B:** Coexpression of $P2X_4$ receptor and ED1 in the periventricular area of the third ventricle (asterisks indicate outline) at E16. **C:** Coexpression of $P2X_7$ and ED1 in the dorsal wall of the third ventricle (asterisks indicate outline) at E16. **D:** Coexpression of $P2X_1$ receptor and microglial cell marker (ED1) in the corpus callosum at E20. **E:** Coexpression of $P2X_4$ receptor and ED1 in the periventicular area of the third ventricle (astetrisks indicate outline) at E20. F: Coexpression of $P2X_7$ and ED1 in the corpus callosum at E20. Scale bars = 100 μm.

$P2X_4$, and $P2X_7$ receptors fitted those expected from the primer pairs (Fig. 4A,B).

RT-PCR Controls

These experiments demonstrated that, on denaturation of the reverse transcriptase, no P2X receptor cDNA could be detected.

DISCUSSION

Microglial cells, the resident macrophages in the brain, are involved in the protection of the integrity of the CNS but, in some instances, are also involved in establishing or exacerbating a number of pathological conditions (Kreutzberg, 1996; Minghetti and Levi, 1998). They are capable of performing a variety of functions such as secretion of cytokines, eicosanoids and free radicals, presentation of the antigen to T lymphocytes and phagocytosis. They are highly responsive to environmental stimuli, one of which may be extracellular ATP. Much pharmacological data has shown that microglial cells express $P2X_7$ receptors and possibly another P2X member, the $P2X_4$ receptor (Ferrari et al., 1996; Haas et al., 1996; Illes et al., 1996; Chessell et al., 1997; Visentin et al., 1999). In particular, studies carried out on mouse cell lines using intracellular Ca^{2+} recording (Ferrari et al., 1996) and patch-clamp methods (Chessell et al., 1997) emphasized the presence of P2Z/$P2X_7$ receptor channels, while patch-clamp studies on rat microglial cells concluded that the fast depolarizing response to ATP was due to P2X receptor channels (Illes et al., 1996). Finally, studies on mouse slice preparations

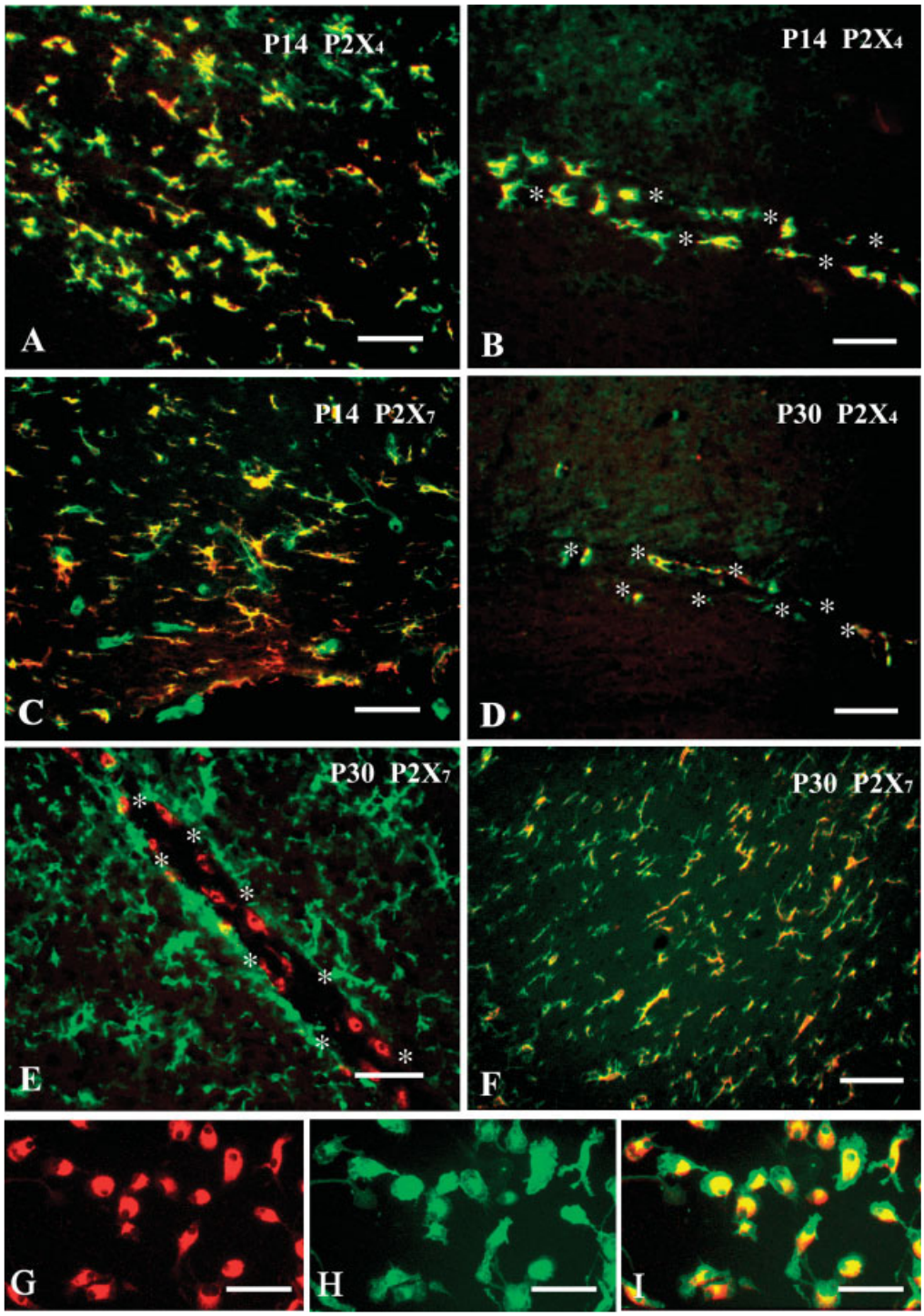

Fig. 2. Coexpression of P2X receptors and ED1 on the microglial cells in the forebrain from P14 to 30, and primary cultured microglial cells were stained with $P2X_4$ antibody and FITC-conjugated IB4. A–E: P2X immunostaining is indicated in green, ED1 immunostaining in red, and colocalization of these in yellow. **A:** Coexpression of $P2X_4$ receptor and the microglial cell marker (ED1) in the corpus callosum at P14. **B:** Coexpression of the $P2X_4$ receptor and ED1 in the cerebral cortex at P14. The positive cells surrounded the blood vessel (indicated by asterisks). **C:** Coexpression of $P2X_7$ and ED1 in the optic chiasma at P14. **D:** Coexpression of the $P2X_4$ receptor and ED1 in the cerebral cortex at P30. The positive cells with $P2X_4$-ir and ED1-ir surrounded blood vessels (indicated by asterisks). **E:** Coexpression of the $P2X_7$ receptor and ED1 in the cerebral cortex at P30. Note that no cells were stained with yellow color meaning that $P2X_7$-ir cells do not express ED1. Asterisks indicate a blood vessel (the cells showing red immunoreactivity are microglia). F-I. P2X immunostaining is in green, IB4 immunostaining is in red and colocalization of these is seen as yellow. **F:** Coexpression of $P2X_7$ and IB4 in the corpus callosum at P30. **G:** $P2X_4$-ir on primary cultured microglial cells. **H:** IB4 on the primary cultured microglial cells. **I:** Colocalization of P2X4 receptors and IB4. Scale bars = 100 μm in A–F; 50 μm in G–I.

envisaged the presence of both a "normal" P2X receptor channel, and a P2Z/$P2X_7$ "pore" responsible for the ATP-induced permeabilization, but detectable only after a sustained treatment with high doses of ATP (Haas et al., 1996). In the present study, $P2X_1$, $P2X_4$, and $P2X_7$ receptor subtypes were shown on the microglial cells in vivo and in vitro by immunocytochemistry and RT-PCR. These results were consistent with the previous pharmacological data that showed $P2X_7$ and possibly $P2X_4$ receptor subtypes on microglial cells (Ferrari et al., 1996; Haas et al., 1996; Illes et al., 1996; Chessell et al., 1997; Visentin, 1999). The $P2X_1$ receptor subtype has been demonstrated to be present on microglial cells in vitro and in vivo by immunocytochemistry and RT-PCR, for the first time in our study. This differed from the previous results from pharmacological studies (Visentin et al., 1999), which claimed that there were no homomeric $P2X_1$ or $P2X_3$ receptors on the primary cultured

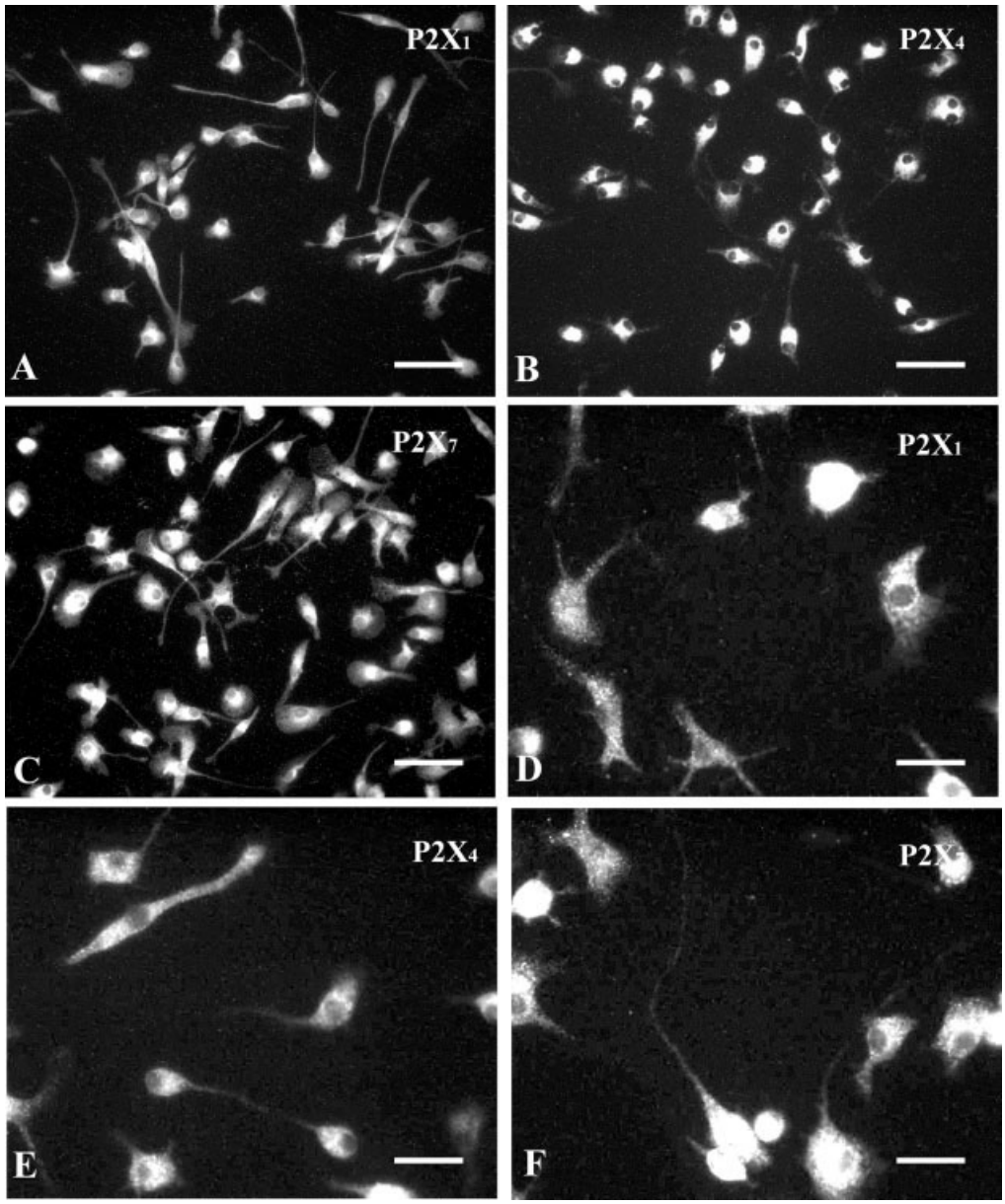

Fig. 3. Expression of P2X receptors on the primary cultured microglial cells and N9 microglial cells. **A:** $P2X_1$ receptor-ir in primary cultured microglial cells. **B:** $P2X_4$ receptor-ir in primary cultured microglial cells. **C:** $P2X_7$ receptor-ir in primary cultured microglial cells. **D:** $P2X_1$ receptor-ir in N9 microglial cells. **E:** $P2X_4$ receptor-ir in N9 microglial cells. F. $P2X_7$ receptor-ir in N9 microglial cells. Scale bars = 100 μm in A–C; 50 μm in D–F.

rat microglial cells. It is possible that the $P2X_1$ receptor expressed on rat microglial cells in early development and in culture, is nonfunctional, but this will need to be resolved by experimental studies.

Generally ED1 labels ameboid-like and activated microglial cells (Domaradzka-Pytel et al., 1999, 2000) and isolectin B4 labels amoeboid-like, activated and resting microglial cells (Perry and Gordon, 1988; Kato et al., 1994; Yagi et al., 1999; Streit, 2002). About 30% of the microglial cells with ED1-ir were $P2X_7$ receptor-ir in the late embryonic and early postnatal forebrain. This means there is selective expression of $P2X_7$ receptors on microglial cells, although it is possible that the microglial cells with or without $P2X_1$-ir or $P2X_7$-ir may be in different functional states.

Another interesting result is that the microglial cells with ED1-ir in the subarachnoid space on the surface of the brain and areas around blood vessels expressed $P2X_4$ receptors, but not $P2X_1$ and $P2X_7$ receptors at all stages examined in this study. This means that in these areas, extracellular ATP can affect microglial cells only via $P2X_4$ receptors. $P2X_1$ receptor-ir microglial cells disappeared at P30 and at this time $P2X_4$ receptor-ir microglial cells appeared to exist only in cells lining the subarachnoid space and the area around the blood vessels. These results demonstrate that there is time and localization specificity in the expression of P2X receptor subtypes in microglial cells.

The microglial precursors (monocytes in blood) migrate into the developing central nervous system and then differentiate into microglial cells from ameboid microglial cells during the late embryonic and early postnatal periods (Perry et al., 1985; Ashwell, 1991; Milligan et al., 1991; Navascués et al., 1996; Dalmau

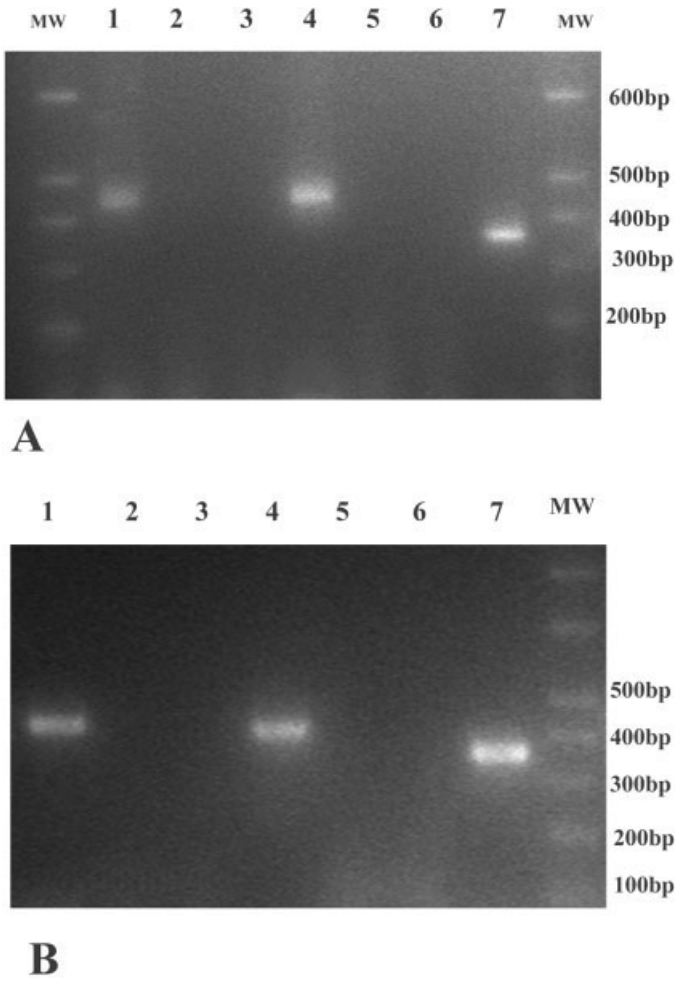

Fig. 4. RT-PCR analysis of P2X receptor mRNAs from primary cultured microglial cells and N9 microglial cells. **A:** RT-PCR results from primary cultured microglial cells. The results showed that $P2X_1$, $P2X_4$ and $P2X_7$ mRNAs are expressed in primary cultured microglial cells. **B:** RT-PCR results from N9 microglial cells. The results showed that $P2X_1$, $P2X_4$, and $P2X_7$ mRNAs are expressed in N9 microglial cells. MW, DNA marker; 1–7, $P2X_1$ to $P2X_7$ cDNAs, respectively.

et al., 1998). The ameboid microglial cells that are positive for ED1 markers are mainly located in the white matter and the area around the ventricles in the early postnatal period. Two weeks postnatally, the number of ameboid microglial cells with ED1 markers decline and disappear after the third week (Domaradzka-Pytel et al., 1999, 2000). The present result demonstrates that the ameboid-like microglial cells with $P2X_1$ and $P2X_4$ receptor-ir appeared at E16 and disappeared between P14 and P30 in the white matter and areas around the ventricles. These results are consistent with previous studies (Domaradzka-Pytel et al., 1999, 2000). Our results suggest that extracellular ATP affects the differentiation and maturation of ameboid microglial cells mainly via $P2X_1$ and $P2X_4$ receptor subunits and that the $P2X_7$ receptor subunit dominates in the resting microglial cells and will mediate extracellular ATP actions in different conditions such as inflammation, injury and degeneration disease.

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