Expression of P2X receptors in rat choroid plexus

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Received 9 February 2005; accepted 11 April 2005

In this study, we have used reverse transcriptase polymerase chain reaction and immunocytochemistry to show that $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptor messenger RNA and protein are expressed in the rat choroid plexus taken from the fourth and lateral ventricles. In some epithelial cells, the apical surfaces were stained more intensely than the basal surfaces. Some of the epithelial cells showed strong staining, others weak or no staining. Double immunostaining with P2X and cytokeratin antibodies or isolectin B4 binding confirmed that P2X receptors were localized in subpopulations of the choroid plexus epithelial cells, rather than the endothelial cells of capillaries. The results suggest that P2X receptors might be involved in the regulation of cerebrospinal fluid composition. *NeuroReport* 16:903–907 © 2005 Lippincott Williams & Wilkins.

Key words: Cerebrospinal fluid; Choroid plexus epithelium; P2X receptors; Purinergic signaling

INTRODUCTION

Increasing evidence shows that extracellular ATP regulates nonneuronal and neuronal cell activities in the brain [1]. These include astrocytes, microglial cells and oligodendrocytes [2]. Cerebral blood vessels also express P2X and P2Y receptors [2]. $P2X_1$, $P2Y_2$ and $P2Y_6$ receptors mediate vasoconstriction of vascular smooth muscle [3,4] while $P2Y_2$ and probably $P2Y_1$ and $P2Y_{12}$ receptors on endothelial cells mediate vasodilation via nitric oxide [5,6]. In addition, the ependyma was reported to express $P2X_7$ receptors [7].

Choroid plexus epithelium is continuous with the ependyma and has the same embryonic origin. The choroid plexus is the major source of cerebrospinal fluid (CSF). The secretory function and hemodynamics of choroidal tissues are regulated by a number of neural and hormonal factors [8]. The aim of this study was to investigate whether P2X receptors are expressed in the choroid plexus, using immunocytochemical and reverse transcriptase polymerase chain reaction (RT-PCR) methods. Colocalization studies were carried out to identify the cell types showing positive expression of P2X receptors using epithelial and endothelial cell markers.

MATERIALS AND METHODS

Animals and tissue preparation: Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with UK Home Office regulations covering Schedule One Procedures and in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. Six adult Wistar rats (250–300 g) were used for this study. 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 brains were removed and choroid plexuses from the fourth and lateral ventricles were dissected out under the microscope. The choroid plexuses were refixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer pH 7.4 overnight and then transferred to 20% sucrose phosphate-buffered saline (PBS) and kept in the solution until they sank to the bottom. Thereafter, the specimens were rapidly frozen by immersion in isopentane at −70°C for 2 min. Sections of 10 μm thickness were cut using a cryostat (Leica, Heerbrugg, Switzerland) and thawed on slides coated with gelatin.

Immunohistochemistry: The development and specificity of the P2X polyclonal antibodies used in this study have been reported previously [9,10]. Tissue sections were washed 3 × 5 min in 0.01 mol/L pH 7.2 PBS. 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 $P2X_7$ (Roche Palo Alto, California, USA) antibodies, diluted 1:500 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight, at 4°C, respectively. Subsequently, the sections were incubated with Cy3-conjugated donkey–anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted 1:300 for P2X antibodies. All incubations and reactions were separated by 3 × 5 min washes in PBS. After P2X immunostaining, sections were incubated with a second primary antibody of either cytokeratin (monoclonal, Sigma Chemical Co., Poole, UK) diluted 1:200 or isolectin B4 [(IB4) fluorescein isothiocyanate (FITC)-conjugated, Sigma] diluted 1:100 in antiserum dilution solution, overnight at 4°C. Subsequently, sections were incubated with FITC-conjugated donkey–anti-mouse IgG (Jackson) diluted 1:200

in antiserum dilution solution, for 1 h at room temperature. All incubations and reactions were separated by 3×10 min washes in PBS.

Control experiments were carried out with $P2X_1$–$P2X_7$ antisera preabsorbed with the relevant cognate peptide.

Photomicroscopy: Images of immunofluorescence labeling were taken with the Leica DC 200 digital camera (Leica) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0). The two channel readings for green and red fluorescence were merged using Adobe Photoshop 5.0.

Reverse transcriptase polymerase chain reaction: Three Wistar rats (250–300 g) were used for RT-PCR experiments. Three sets of total RNA were extracted from choroid plexuses dissected from the fourth and lateral ventricles using the SV Total RNA Isolation System (Promega, Madison, Wisconsin, USA). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech, Bucks, UK). Reverse transcription was performed using the Moloney murine leukemia virus reverse transcriptase. Primer sequences for $P2X_{1-7}$ [11] 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. Each sample was further treated with amplification Grade DNase I (Sigma) to remove any residual DNA 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. Sequence specific primers were then added to the reaction mixtures. The following PCR cycling parameters were then used: 95°C for 45 s, 60°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 2.0% agarose gel containing ethidium bromide and observed under ultraviolet illumination. RT-PCR results for all tissues were confirmed by repetition with three separate RNA samples.

RESULTS

Immunoreactivity for six members of the P2X receptor family ($P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$) was seen in the epithelium of choroid plexus taken from the fourth and lateral ventricles; $P2X_3$ staining was not observed in these cells. The pattern of immunoreactivity for these six P2X receptors subtypes was similar; positive staining was seen in the cytoplasm but not in the nuclei. The majority of epithelial cells were strongly stained by all six antibodies. In some cells, the apical surfaces appeared to be stained more intensely than the basal surfaces. Some epithelial cells showed heavy staining while other adjacent cells were weakly stained (Fig. 1).

In order to establish which cell type(s) in the choroid plexus were stained by P2X receptor antibodies, double immunostaining was carried out. The cells with P2X immunoreactivities were also labeled by the cytokeratin antibody, a marker for epithelial cells (Fig. 2a–d), although some cells stained for cytokeratin but not for $P2X_3$. IB4, which is a marker for the endothelial cells, did not show P2X receptor immunoreactivity (Fig. 2e and f).

In control immunostaining experiments, no staining was observed in those specimens incubated with antibody solutions pre-absorbed with P2X peptides.

The RT-PCR results showed that $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$, but not $P2X_3$ receptor messenger RNA (mRNA), were expressed in the choroid plexuses (Fig. 3). Control experiments were conducted by denaturing the reverse transcriptase (95°C for 10 min) before the RT-PCR reaction. These experiments demonstrated that, on denaturation of the reverse transcriptase, no P2X receptor cDNA could be detected.

DISCUSSION

In this study, we used RT-PCR and immunocytochemistry techniques to show that $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ mRNA and protein were expressed in the choroid plexus epithelium of the rat, but not in the capillary endothelium of the connective tissue core of the choroid plexus. Cytokeratin is an epithelial cell marker that is expressed in the choroid plexus epithelial cells [12]. The cells with P2X receptor immunoreactivity were also labeled by the cytokeratin antibody and these cuboidal cells were localized to the surface of the choroid plexus. These data confirmed that the cells with P2X receptor immunoreactivity were choroid plexus epithelial cells. IB4 is used as a marker for endothelial cells in blood vessels [13]. In this study, the cells with FITC-conjugated IB4 were not labeled by any of the P2X receptor subtype antibodies. Our results raise the possibility that extracellular ATP may regulate, via P2X receptors, the function of choroid plexus epithelial cells but not endothelial cells.

The epithelial cells of the choroid plexus secrete cerebrospinal fluid (CSF), by a process that involves the movement of Na^+, Cl^- and HCO_3^- from the blood to the ventricles of the brain. This creates the osmotic gradient, which drives the secretion of H_2O [14]. Two potassium channels have been reported to have a role in maintaining the membrane potential of the epithelial cells, and also in regulating the transport of K^+ across the epithelium [15,16]. An inward-rectifying anion channel has also been identified, which is closely related to ClC-2 channels, and has a significant HCO_3^- permeability. This channel is expressed in the apical membrane of the epithelium in which it may play an important role in CSF secretion [17,18]. P2X receptors have an intrinsic ion channel that increases the permeability of the plasma membrane to Na^+, K^+, Ca^{2+} and some anions [19]. In this study, we observed that in some cells, the apical surfaces of the choroid plexus epithelium were more strongly stained than the basal surfaces. This suggests that P2X receptors may be involved in the secretion of CSF. In a previous study, the presence of moderate expression of mRNA for $P2Y_4$ receptors was found in the adult rat choroid plexus [20], again suggesting that ATP may play a role in the function of the choroid plexus, although no direct evidence exists yet to support this speculation.

Two types of epithelial cells, 'dark and light', have been reported in choroidal epithelial cells of several mammalian species [21–24]. It has been suggested that these differences in appearance of cells may represent different states of cell

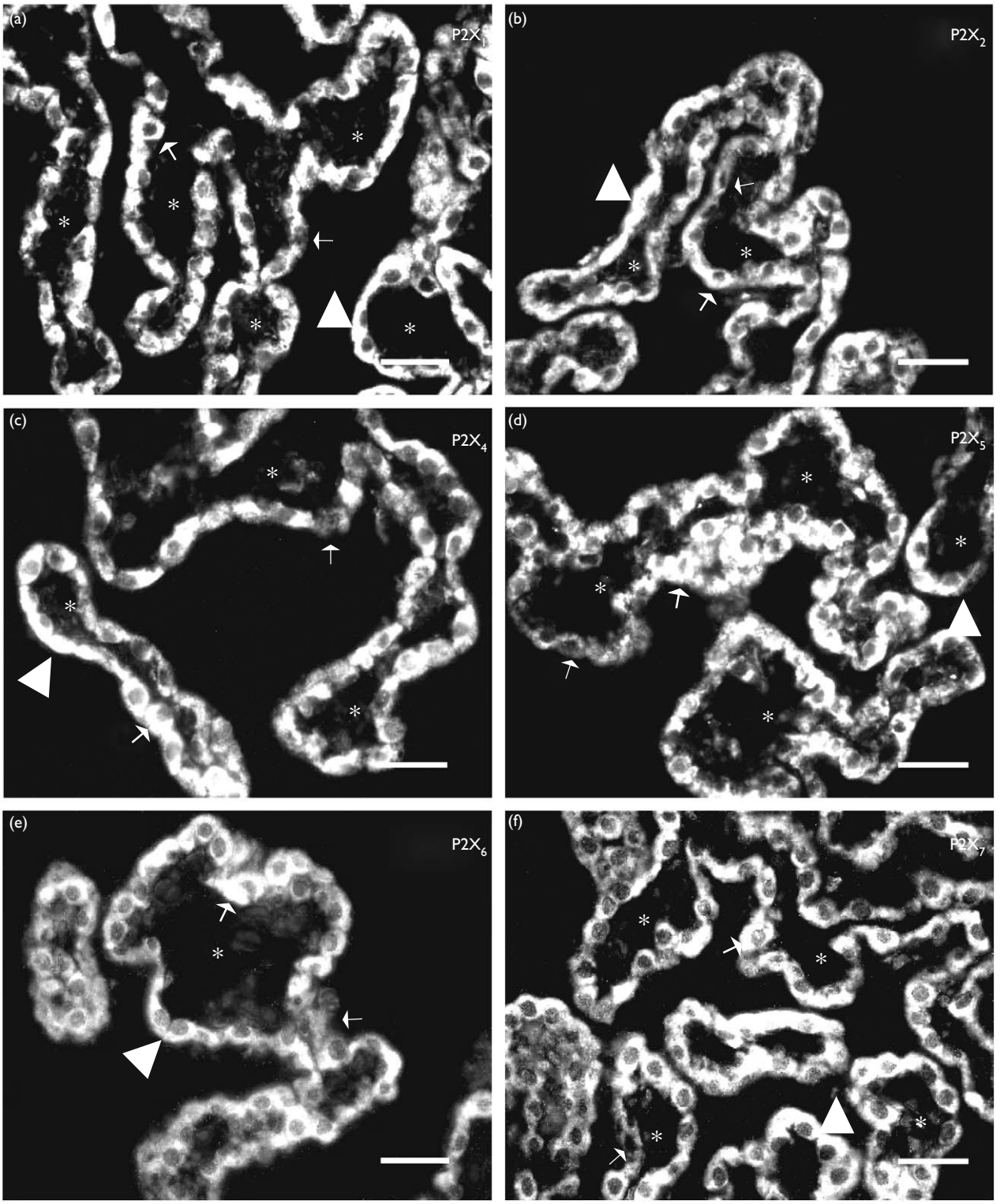

Fig. 1. Expression of the P2X receptor in the choroid plexus epithelial cells of the adult rat. (**a**) $P2X_1$ receptor immunoreactivity. (**b**) $P2X_2$ receptor immunoreactivity. (**c**) $P2X_4$ receptor immunoreactivity. (**d**) $P2X_5$ receptor immunoreactivity. (**e**) $P2X_6$ receptor immunoreactivity. (**f**) $P2X_7$ receptor immunoreactivity. Some cells show intense staining (large arrows) while adjacent cells often show weak staining (small arrows). Arrowheads show the areas of some cells where the positive signals were stronger on apical surfaces than on basal surfaces. Asterisks show the position of connective tissue core in the choroid plexus. All scale bars=100 μm.

hydration, but the possibility that they may be fixation artifacts was also raised [21]. More recently it has also been suggested that they may represent different physiological conditions [25,26]. Dark cells in mice choroid plexus first appear at E14 and in mature mice constitute about 12% of the choroidal epithelials [23]. It is possible that the cells

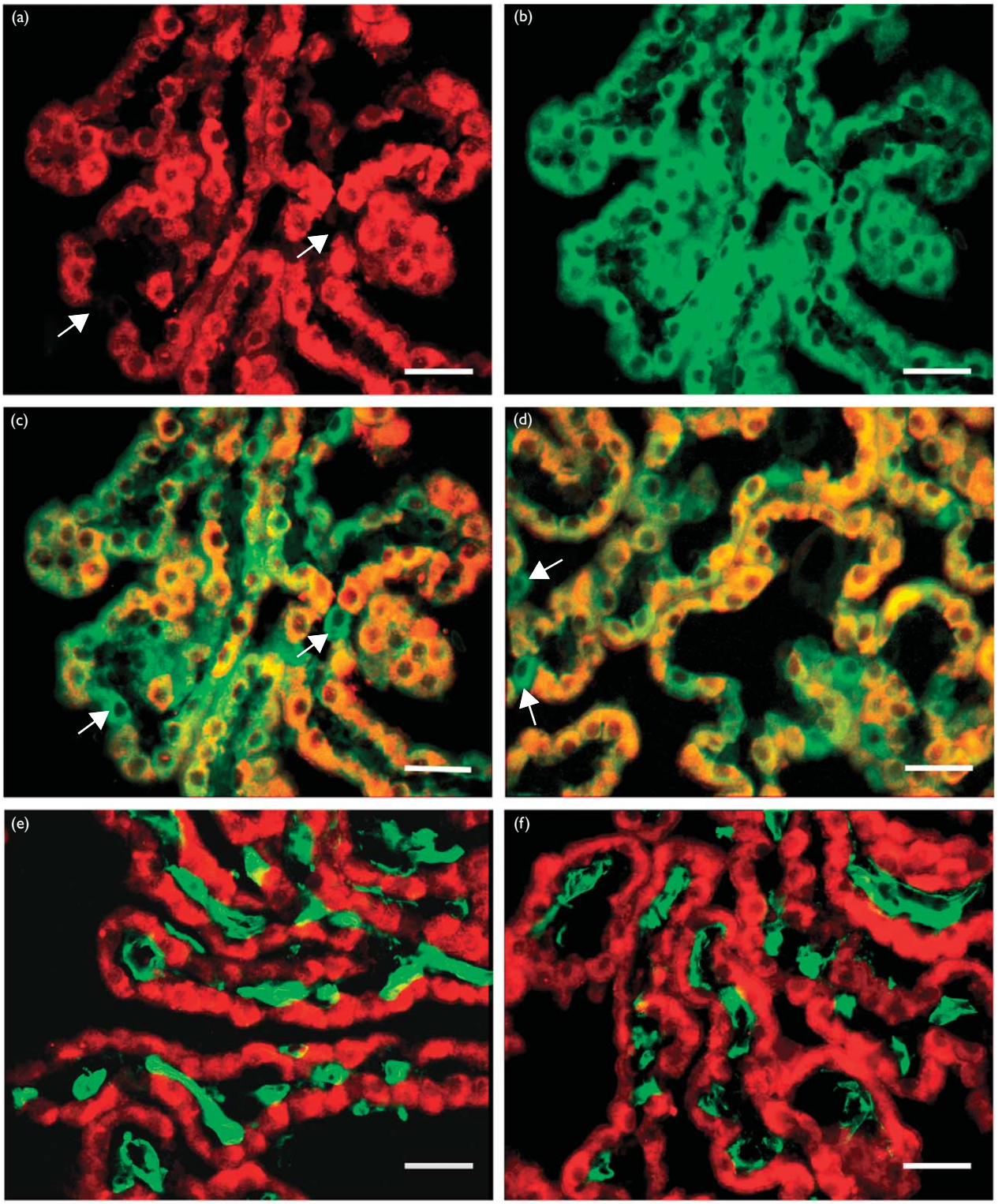

Fig. 2. Coexpression of P2X receptors with cytokeratin and isolectin B4 (IB4) in the choroid plexus of the adult rat. (**a**) $P2X_1$ receptor immunoreactivity (red) in the choroid plexus (arrows indicate negatively stained cells). (**b**) Cytokeratin immunoreactivity (green) in the choroid plexus from the same field as (a). (**c**) Colocalization of $P2X_1$ and cytokeratin immunoreactivity (yellow-orange). Note that some cells were positive for cytokeratin but not for the $P2X_3$ receptor (arrows). (**d**) Coexpression of $P2X_4$ receptor and cytokeratin in the choroid plexus. Note that the choroid epithelial cells are double-labeled with $P2X_4$ receptors and cytokeratin antibodies. Arrows indicate cytokeratin-positive cells that are not $P2X_4$-immunoreactive. (**e**) Coexpression of $P2X_5$ receptors and IB4. (**f**) Coexpression of the $P2X_7$ receptor and IB4. Note that in (e) and (f) no coexisting cells are found with P2X receptor immunoreactivity and IB4 binding. All scale bars=100 μm.

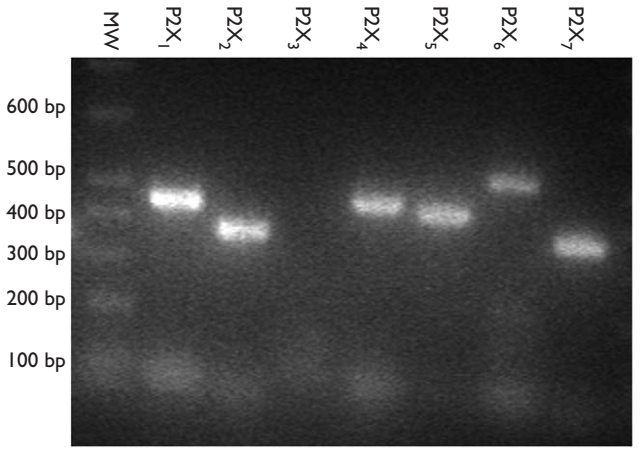

Fig. 3. Reverse transcriptase polymerase chain reaction analysis of P2X receptor messenger RNAs (mRNAs) from adult rat choroid plexuses. MW=DNA marker. $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ mRNAs were expressed, but not $P2X_3$.

showing weak immunostaining for P2X receptors in our experiments are the dark cells, but the functional significance of the lower expression of P2X receptors in these cell subpopulations is not yet clear. In conclusion, the results here presented raise the possibility that P2X receptors might be involved in the regulation of cerebrospinal fluid composition.

REFERENCES

1. Burnstock G. Purinergic receptors in the nervous system. In: Schwiebert EM (ed.) *Current Topics in Membranes, Vol. 54. Purinergic Receptors and Signalling*. San Diego: Academic Press; 2003. pp. 307–368.
2. Burnstock G, Knight GE. Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* 2004; **240**: 31–304.
3. von Kügelgen I, Starke K. Evidence for two separate vasoconstriction mediating nucleotide receptors, both distinct from the P2X-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn-Schmiedeberg's Arch Pharmacol* 1990; **341**:538–546.
4. Malmsjö M, Hou M, Pendergast W, Erlinge D, Edvinsson L. The stable pyrimidines UDPβS and UTPγS discriminate between contractile cerebrovascular P2 receptors. *Eur J Pharmacol* 2003; **458**:305–311.
5. Simon J, Vigne P, Eklund KM, Michel AD, Carruthers AM, Humphrey PP *et al*. Activity of adenosine diphosphates and triphosphates on a P2Y(T) -type receptor in brain capillary endothelial cells. *Br J Pharmacol* 2001; **132**:173–182.
6. Horiuchi T, Dietrich HH, Hongo K, Dacey RG. Comparison of P2 receptor subtypes producing dilation in rat intracerebral arterioles. *Stroke* 2003; **34**:1473–1478.
7. Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North RA, Buell G. Tissue distribution of the $P2X_7$ receptor. *Neuropharmacology* 1997; **36**: 1277–1283.
8. Nilsson C, Lindvall-Axelsson M, Owman C. Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Res Rev* 1992; **17**:109–138.
9. Xiang Z, Bo X, Burnstock G. Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. *Neurosci Lett* 1998; **256**:105–108.
10. Ogleby IB, Lachnit WG, Burnstock G, Ford APDW. Subunit specificity of polyclonal antisera to the carboxyl terminal regions of P2X receptors $P2X_1$ through $P2X_7$. *Drug Dev Res* 1999; **47**:189–195.
11. Shibuya I, Tanaka K, Hattori Y, Uezono Y, Nobuya H, Noguchi J *et al*. Evidence that multiple P2X purinoceptors are functionally expressed in rat supraoptic neurons. *J Physiol* 1999; **514**:351–367.
12. Sarnat HB. Histochemistry and immunocytochemistry of the developing ependyma and choroid plexus. *Microsc Res Technique* 1998; **41**:14–28.
13. Lin SS, Parker W, Everett ML, Platt JL. Differential recognition by proteins of alpha-galactosyl residues on endothelial cell surfaces. *Glycobiology* 1998; **8**:433–443.
14. Speake T, Whitwell C, Kajita H, Majid A, Brown PD. Mechanisms of CSF secretion by the choroid plexus. *Microsc Res Technique* 2001; **52**:49–59.
15. Zlokovic BV, Mackic JB, Wang L, McComb JG, McDonough A. Differential expression of Na,K-ATPase α and β subunit isoforms at the blood-brain barrier and the choroid plexus. *J Biol Chem* 1993; **268**:8019–8025.
16. Rizzolo LJ. Polarization of the Na^+,K^+-ATPase in epithelia derived from the neuroepithelium. *Int Rev Cytol* 1999; **185**:195–235.
17. Frankel H, Kazemi H. Regulation of CSF composition-blocking chloride-bicarbonate exchange. *J Appl Physiol* 1983; **55**:177–182.
18. Deng QS, Johanson CE. Stilbenes inhibit exchange of chloride between blood, choroid plexus and the cerebrospinal fluid. *Brain Res* 1989; **510**:183–187.
19. Schwiebert EM, Zsembery A. Extracellular ATP as a signaling molecule for epithelial cells. *Biochim Biophys Acta* 2003; **1615**:7–32.
20. Webb TE, Henderson DJ, Roberts JA, Barnard EA. Molecular cloning and characterization of the rat $P2Y_4$ receptor. *J Neurochem* 1998; **71**:1348–1357.
21. Dohrmann GJ. Dark and light epithelial cells in the choroid plexus of mammals. *J Ultrastruct Res* 1970; **32**:268–273.
22. van Deurs B, Møller M, Amtorp O. Uptake of horseradish peroxidase from CSF into the choroid plexus of the rat, with special reference to transepithelial transport. *Cell Tissue Res* 1978; **62**:168–180.
23. Sturrock RR. A morphological study of the development of the mouse choroid plexus. *J Anat* 1979; **129**:777–793.
24. Joakim Ek C, Habgood MD, Dziegielewska KM, Saunders NR. Structural characteristics and barrier properties of the choroid plexuses in developing brain of the opossum *(Monodelphis Domestica)*. *J Comp Neurol* 2003; **460**:451–464.
25. Shuman CS, Bryan JH. Comparative quantitative ultrastructural studies of the choroidal epithelium of hydrocephalic (hpy/hpy) and normal mice, and the effect of stress induced by water deprivation. *Anat Anz* 1991; **173**:33–44.
26. Johanson CE, Preston JE, Chodobski A, Stopa EG, Szmydynger-Chodobska J, McMillan PN. AVP V1 receptor-mediated decrease in Cl^- efflux and increase in dark cell number in choroid plexus epithelium. *Am J Physiol* 1999; **276**:C82–C90.

Acknowledgement: The authors thank Dr Chrystalla Orphanides for editorial assistance.