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REGULAR ARTICLE

Andrzej Loesch · Geoffrey Burnstock

Electron-immunocytochemical localization of $P2X_1$ receptors in the rat cerebellum

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Abstract The distribution of the $P2X_1$ subtype of purinoceptors associated with the extracellular activities of ATP was studied in the rat cerebellum at the electron-microscope level. Receptors were labelled with peroxidase-antiperoxidase and the avidin-biotin-peroxidase complex for immunocytochemistry. Immunoreactivity to $P2X_1$ receptors was localized in subpopulations of synapses between varicosities of parallel fibres of granule cells and dendritic spines of Purkinje cells. Unlabelled varicosities of parallel fibres formed asymmetric synapses with labelled dendritic spines, whereas labelled varicosities of parallel fibres formed asymmetric synapses with unlabelled dendritic spines. $P2X_1$ immunoreactivity was also localized in some astrocyte processes. The functional significance of these findings is discussed.

Key words $P2X_1$ receptor · Ultrastructure · Cerebellum · Rat (Wistar)

Introduction

It is now generally accepted that extracellular purines, such as adenosine and adenosine 5'-triphosphate (ATP), act as mediators and regulators of cell function through receptors (Burnstock 1997). An initial classification of purine receptors distinguished between adenosine receptors (P1) and ATP receptors (P2; Burnstock 1978). P2 receptors have now been divided into two major families, including $P2X_{1–7}$ and $P2Y_{1–8}$ subtypes according to their molecular identity and their intracellular signal transduction pathways (Abbracchio and Burnstock 1994; Burnstock and King 1996); P2X receptors are ligand-gated cation channels involved in neurotransmission, whereas P2Y (G protein-coupled) receptors are involved in slower events such as exocrine and endocrine secretion (see Burnstock 1996).

In situ hybridization and a few immunocytochemical studies have previously localized the P2X receptors in various mammalian tissues, including the central and peripheral nervous systems at the light-microscopic level (Chen et al. 1995; Kidd et al. 1995; Collo et al. 1996; Kanjhan et al. 1996; Vulchanova et al. 1996, 1997; Chan et al. 1998; Nori et al. 1998). So far, there has been only one ultrastructural study of purinoceptors demonstrating the localization of $P2X_4$ immunoreactivity in the glomeruli of the olfactory bulb, the substantia gelatinosa of the spinal cord and in the Purkinje cell perikarya of the cerebellar cortex of adult male Sprague-Dawley rats (Lê et al. 1998). As far as we are aware, there has been no study showing immunoreactivity to the $P2X_1$ receptor subtype in the cerebellum. In the present study, we reveal for the first time the electron-immunocytochemical localization of $P2X_1$ receptors in rat cerebellum.

Materials and methods

Tissues

Eight adult (3- to 4-month-old) male Wistar rats were anaesthetized with sodium pentobarbitone (60 mg/kg i.p., Sagatal, RMB Animal Health, Harlow, UK) and perfused through the heart (left ventricle) with fixative containing 4% paraformaldehyde, 0.15% glutaraldehyde and 0.05% picric acid in 0.1 M phosphate buffer, pH 7.4. The brains were removed, placed in the same fixative for 5 h at 4°C, transferred to 4% paraformaldehyde and stored overnight at 4°C. The following day, the brains were rinsed in phosphate buffer for several hours (at 4°C) and then transferred to 0.05 M TRIS-buffered saline, pH 7.6 (TBS, High Wycombe, UK). Vertical (sagittal) sections of 50–60 μm through the cerebellum were cut on a Vibratome and collected in TBS. After being washed in TBS, the sections were exposed for 45 min to 0.3% hydrogen peroxide in 33% methanol for blocking endogenous peroxidases, washed in TBS and pro-

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A. Loesch (✉)
Department of Anatomy and Developmental Biology,
University College London, Gower Street, London WC1E 6BT, UK
Tel.: +44 171 504 2215; Fax: +44 171 380 7349

G. Burnstock
Autonomic Neuroscience Institute,
Royal Free Hospital School of Medicine, Rowland Hill Street,
London NW3 2PF, UK

cessed for the pre-embedding electron immunocytochemistry of $P2X_1$ antibody, by means of the peroxidase-antiperoxidase complex (PAP) and ExtrAvidin peroxidase-conjugate (ABC) methods at room temperature.

PAP procedure

In brief, sections/vibroslices were exposed for 1.5 h to heat-treated (55°C for 1 h) 10% normal goat serum (Nordic Immunology, Tilburg, The Netherlands) in TBS containing 0.05% thimerosal (Sigma, Poole, UK), rinsed in TBS and then incubated for 65 h with a rabbit polyclonal antibody to $P2X_1$ (1.5–3.0 µg antibody/ml TBS containing 5% normal goat serum and 0.05% thimerosal). After being rinsed in TBS (3×30 min), the specimens were then incubated for 24 h with a goat anti-rabbit immunoglobulin G serum (Biogenesis, Bournemouth, UK) diluted 1:80 in TBS containing 1% normal goat serum and 0.05% thimerosal. After being rinsed in TBS (3×30 min), specimens were incubated for 5 h with a rabbit PAP complex (DAKO, Glostrup, Denmark) diluted 1:80 in TBS. Specimens were rinsed in TBS (3×30 min) and then treated for 20 min with 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% hydrogen peroxide. Following the immunoprocedure, specimens were rinsed in water and 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide for 45 min, rinsed in cacodylate buffer, dehydrated in a graded series of ethanol and propylene oxide and embedded in Durcupan (Sigma). Ultrathin sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope.

ABC procedure

The steps of the ABC immunoprocedure were generally similar to those of the PAP method. However, the main steps included: incubation with a heat-treated 10% normal donkey (or horse) serum (Jackson ImmunoResearch Laboratories, West-Grave, Pa., USA; distributor: Stratech Scientific, Luton, UK); incubation with a rabbit polyclonal antibody to $P2X_1$ (1.5–3.0 µg antibody/ml TBS containing 5% normal donkey serum and 0.05% thimerosal); incubation with a biotin-conjugated donkey anti-rabbit immunoglobulin G (H+L) serum (Jackson ImmunoResearch Laboratories) diluted 1:500 in TBS containing 1% normal donkey serum and 0.05% thimerosal; incubation with ExtrAvidin-horseradish peroxidase conjugate (Sigma) diluted 1:1500 in TBS. After exposure to DAB and osmication, the specimens were dehydrated and embedded in Durcupan (Sigma). The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope. Identification of the neuronal/glial elements examined was primarily based on the description in detail of the ultrastructural organization of the rat cerebellum (Palay and Chan-Palay 1974).

Controls for immunocytochemistry

The rabbit polyclonal antibody to $P2X_1$ used in this study was generated and characterized by Roche Bioscience, Palo Alto, USA. In brief, the immunogen used was a synthetic peptide corresponding to the peptide sequence of 15 amino acids located within the C-terminus of cloned $P2X_1$ receptor proteins from rat vas deferens (NH_2-ATSSTLGLQENMRTS-COOH, residues 385–399, Genbank Association no. X80477), covalently linked to keyhole limpet haemocyanin. The antibody was raised in New Zealand rabbits by multiple monthly injections with the immunogen (performed by Research Genetics, Huntsville, Ala., USA). IgG fractions were isolated from the immune sera and from pre-immune controls by chromatography on DEAE Affigel-Blue (Bio-Rad, Hemel Hempstead, UK) or following the method of Harboe and Inglid (1973). The specificity of the antibody was verified by Western blot analysis of membrane preparations from a native tissue source (rat vas deferens) and recombinant CHO-K1 cells expressing the cloned $P2X_1$ receptor (the C-terminal 15-amino-acid sequences of the rat and human $P2X_1$ receptor proteins are identical to one another but different from the sequences present in other P2X receptor subtypes). Immunoblotting studies showed that anti-$P2X_1$ antibody specifically recognized the recombinant $P2X_1$ receptor expressed in CHO-K1 cells (60 kDa band) and that preabsorption of the antibody with an excess of the synthetic peptide used for the generation of the antibody eliminated the immunoreactivity/band (see Chan et al. 1998). In the present study, preabsorption of $P2X_1$ antibody with the antigen (synthetic peptide used for the generation of the antibody) at a concentration of 5 µg/ml diluted antibody (5 µg peptide: 3 µg antibody) was sufficient to abolish immunostaining in the PAP study. Furthermore, no immunolabelling was observed when the $P2X_1$ antibody was omitted from the incubation medium and/or replaced with non-immune normal goat serum and non-immune normal rabbit serum (both from Nordic Immunology, Tilberg, The Netherlands), and when the goat anti-rabbit immunoglobulin G serum (Biogenesis) was omitted.

Results

Both the PAP and ABC methods revealed immunoreactivity to $P2X_1$ in rat cerebellum (Figs. 1–3). The most prominent immunoreactivity was observed in the molecular layer of the cerebellar cortex, where it labelled a small subpopulation of spines/thorns of Purkinje cell dendrites (Fig. 1) and parallel fibres of granule cells (Fig. 2). The immuno-positive dendritic spines of Purkinje cells synapsed with immuno-negative axon varicosities of granule cells (Fig. 1a, b). In some labelled dendritic spines, the pronounced $P2X_1$ immunoreactivity was concentrated at the postsynaptic density of the junction, leaving the other region of the dendrite less well labelled (Fig. 1c). The $P2X_1$-positive profiles of parallel fibers were scattered between the immuno-negative fibres (Fig. 2a) or were located close to the immuno-negative axo-dendritic synapses formed by a varicosity of parallel fibre and dendritic spines (Fig. 2b). Immuno-positive varicosities (*en passant*) of parallel fibres containing spherical synaptic vesicles were also observed either lying close to the dendritic spines (Fig. 2c) or forming asymmetric synapses with immuno-negative dendritic spines (Fig. 2d). $P2X_1$ immunoreactivity was also identified in the neuroglia cell processes (astrocytes) both in the granular and more frequently in the molecular layer (Fig. 3a-c); the whole bodies of neuroglia cells were never labelled (Fig. 3a). For example, immuno-positive processes of glial cells were seen in the vicinity of Purkinje cells (Fig. 3a) or ascending axons of basket cells in the lower molecular layer (Fig. 3b, c). Neither neuronal nor glial cell profiles showed $P2X_1$ immunoreactivity in immunocytochemical control preparations (data not shown).

Discussion

The present study describes the presence of $P2X_1$ receptors associated with subpopulations of neuronal profiles, viz. parallel fibres of granule cells and dendritic spines of Purkinje cells in the molecular layer, and neuroglia (astrocyte) processes in both granular and molecular layers of the rat cerebellum. These data suggest that purines

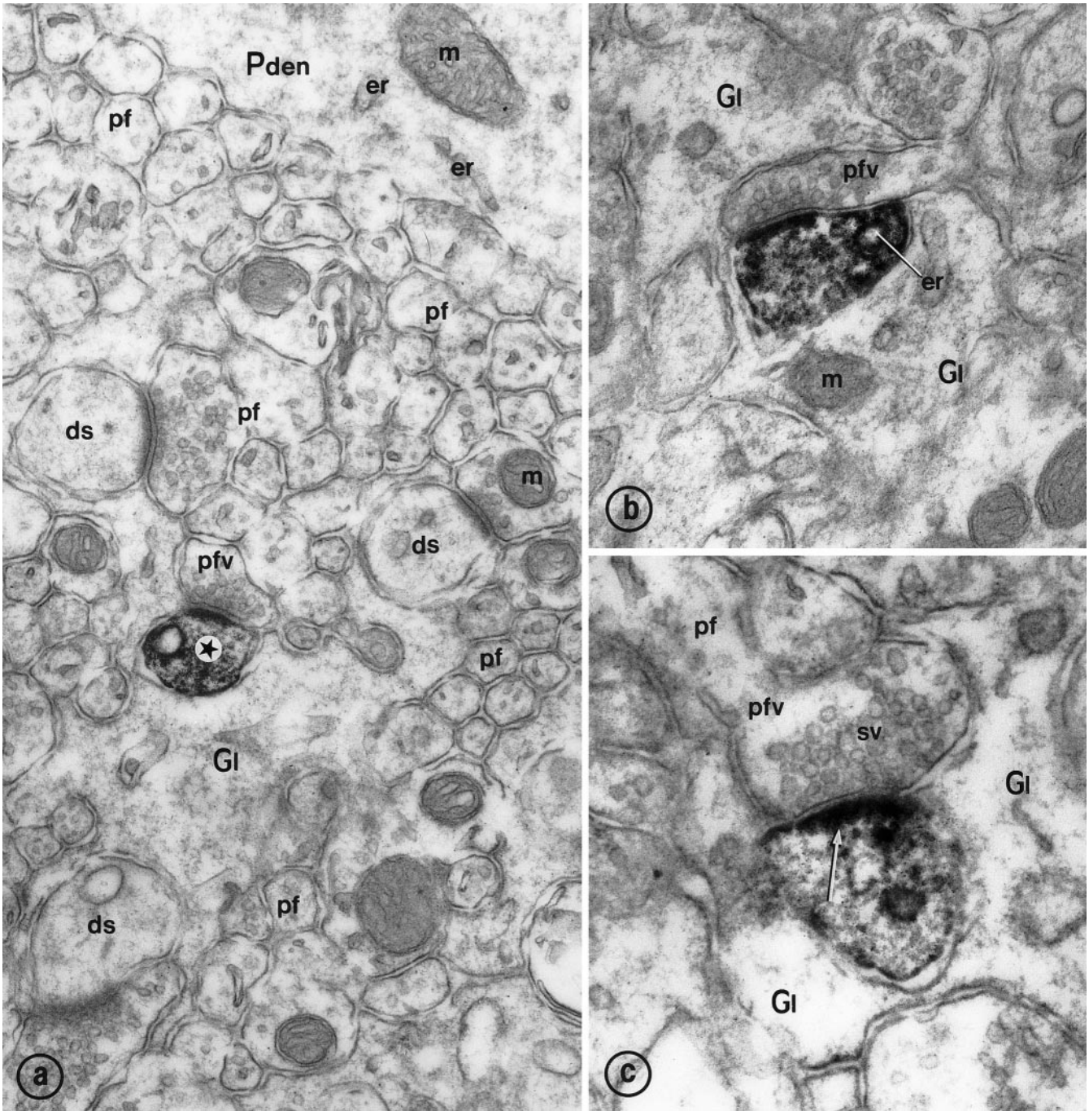


Fig. 1 Molecular layer of rat cerebellum processed for PAP (**a, b**) and ABC (**c**) immunocytochemistry showing $P2X_1$-positive dendritic spines of Purkinje cells involved in the formation of asymmetric axo-dendritic synapses. **a** Note the $P2X_1$-negative varicosity of the parallel fibre (*pfv*) of the ascending granule cell axon upon the $P2X_1$-positive dendritic spine (*black star*); the varicosity contains spherical agranular synaptic vesicles. At least three $P2X_1$-negative postsynaptic dendritic spines (*ds*) and $P2X_1$-negative clusters of parallel fibers (*pf*) can also be seen [*Pden* thin dendrite (part of spiny branchlets) of a Purkinje cell, *m* mitochondria, *er* agranular endoplasmic reticulum, *Gl* neuroglia processes]. ×44 000. **b** Cisternae of endoplasmic reticulum (*er*) can be seen within the $P2X_1$-positive postsynaptic dendritic spine. ×56 000. **c** A $P2X_1$-positive postsynaptic dendritic spine displaying labelling mostly at the postsynaptic density (*arrow*) (*sv* spherical synaptic vesicles). ×97 000

may have physiological actions in the cerebellum of the rat. It is now well recognized that ATP acts as a fast excitatory neurotransmitter; when released presynaptically, it causes a fast excitatory postsynaptic/postjunctional potential both in central and peripheral neurons and in smooth muscle (Sneddon and Burnstock 1984; Sneddon and Westfall 1984; Dunn and Blakely 1988; Edwards et al. 1992; Evans and Surprenant 1992; Evans et al. 1992; Galligan and Bertrand 1994; see also Burnstock 1996). This fast transmission is mediated by ATP receptors (P2X) located postsynaptically. Since P2X receptors have been localized on the dendritic spines of Purkinje cells where they form synapses with granule cell varicosities,

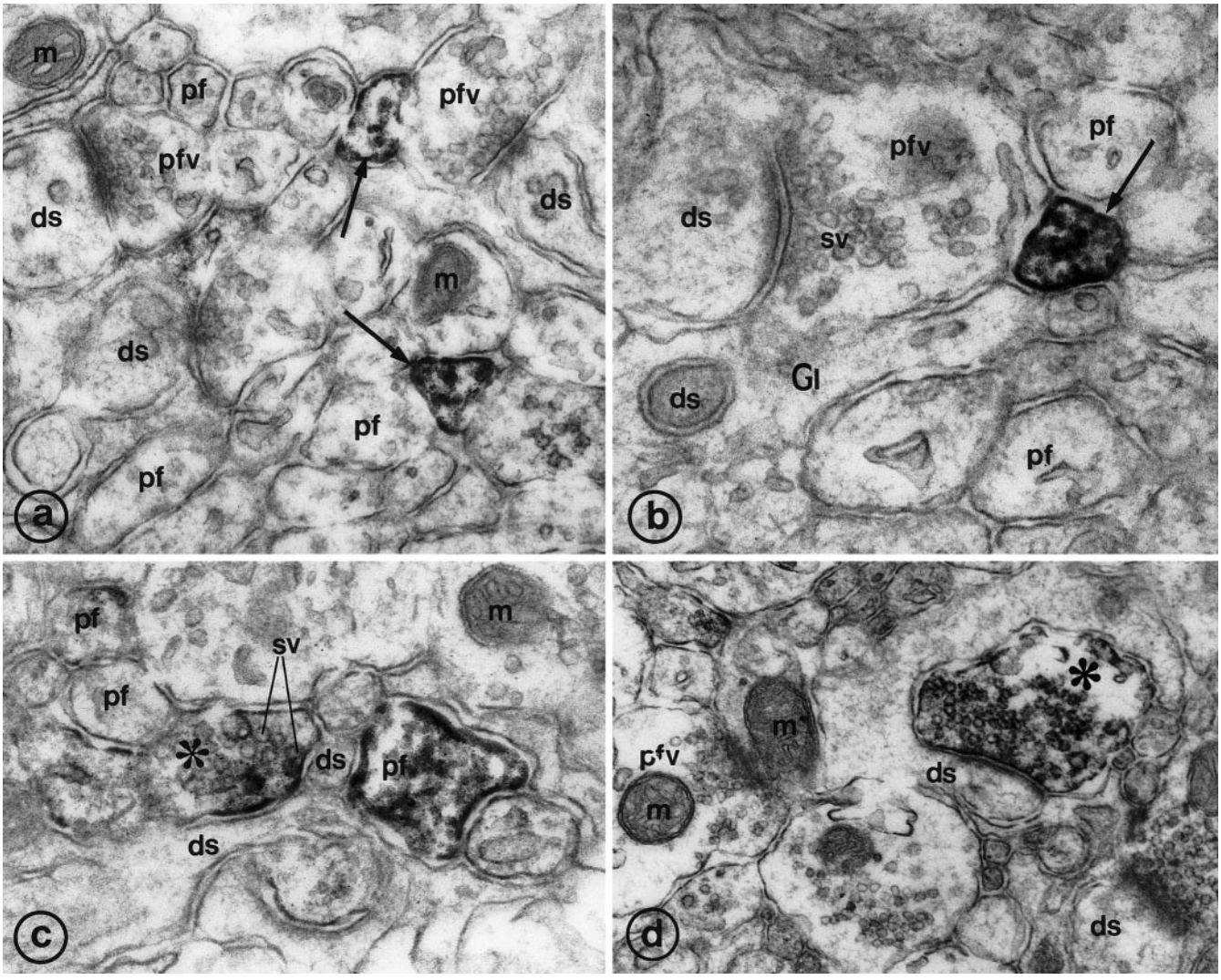
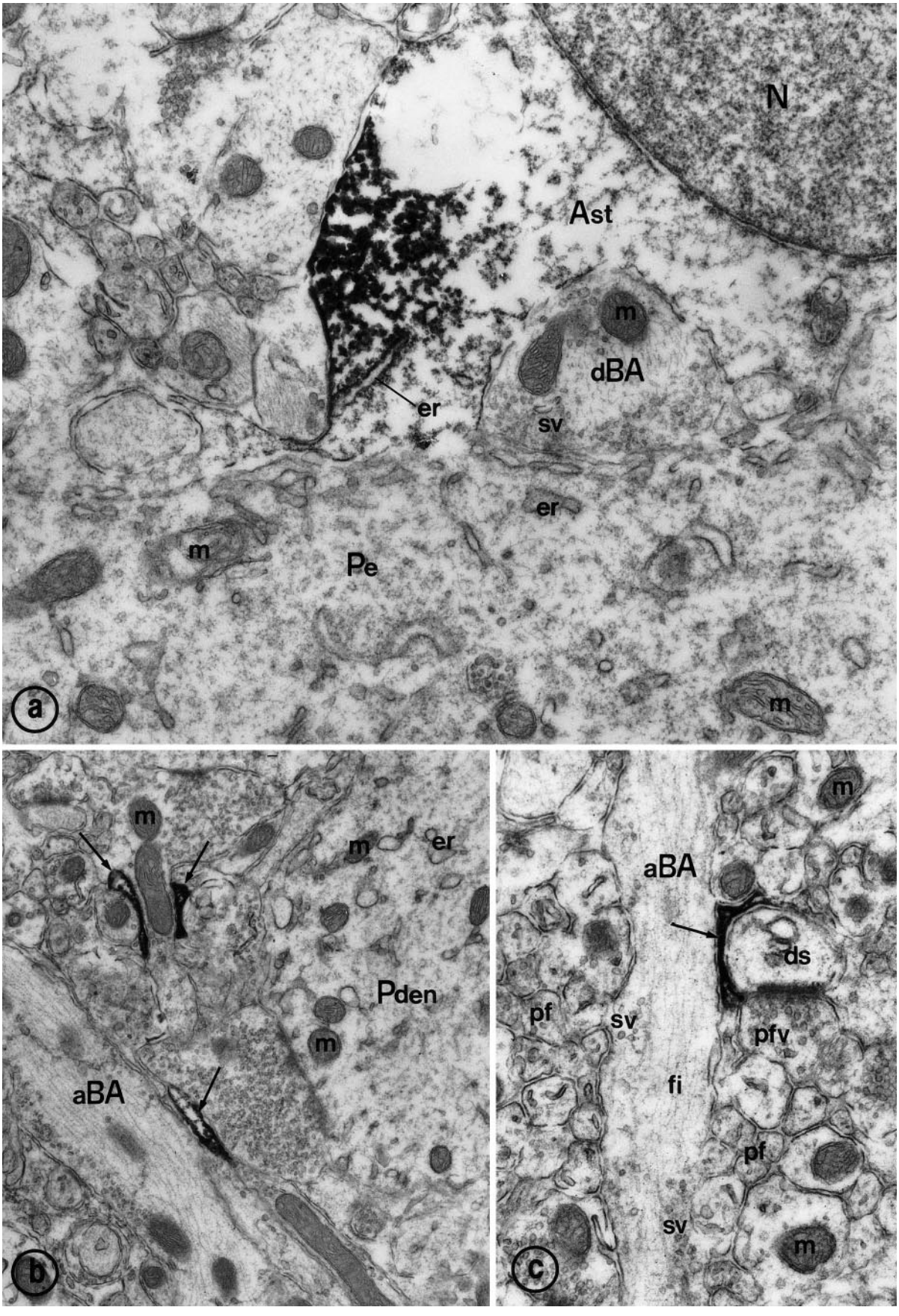


Fig. 2 Molecular layer of rat cerebellum processed for PAP (**a–c**) and ABC (**d**) immunocytochemistry showing $P2X_1$-positive and $P2X_1$-negative parallel fibres (*pf*). **a** Note two $P2X_1$-positive fibres (*arrows*) among the $P2X_1$-negative fibres (*pfv* varicosity of parallel fibres, *ds* dendritic spines, *m* mitochondria). ×59 500. **b** A $P2X_1$-positive parallel fibre close to the axo-dendritic synapse (*sv* spherical synaptic vesicles, *Gl* neuroglia processes). ×70 000. **c** Note the spherical synaptic vesicles in a fragment of a $P2X_1$-positive varicosity (*asterisk*) of a parallel fibre. ×72 500. **d** Note the $P2X_1$-positive varicosity (*asterisk*) of a parallel fibre forming an asymmetric synapse upon a $P2X_1$-negative dendritic spine. ×24 600

this suggests an involvement of ATP as a transmitter or more likely as a co-transmitter at this site. Immunohistochemical localization of ecto-5'-nucleotidase in cultures of cerebellar granule cells adds indirect support for a role for ATP as an extracellular messenger for these neurons (Maienschein and Zimmermann 1996). $P2X_1$ immunoreactivity appears to be present in a subpopulation of parallel fibres suggesting that purines (ATP) may also have presynaptic actions, perhaps modulating transmitter release from the neurons. There have been several reports of ATP actions in the cerebellum. Studies of the single-channel properties of P2X receptors in rat cerebellar slices suggest that the receptor subtypes on Purkinje cells are $P2X_4$ and/or $P2X_6$, either as homomeric or heteromeric forms (Halliday and Gibb 1997). P2Y metabotropic re-

Fig. 3 Rat cerebellum processed for PAP (**a, b**) and ABC (**c**) immunocytochemistry showing $P2X_1$-positive processes of neuroglia cells. **a** Note an astrocyte (*Ast*) in the vicinity of a Purkinje cell soma (*Pe*) displaying a cluster of immunoprecipitate that is limited to the peripheral region of the cytoplasm (*N* nucleus, *er* endoplasmic reticulum, *dBA* descending basket axons contacting a Purkinje cell, *sv* synaptic vesicles, *m* mitochondria). ×28 000. **b** Low-power electron micrograph of the neuropile of the lower molecular layer showing labelled neuroglia-like processes (*arrows*). An ascending basket cell axon (*aBA*) and a dendrite of a Purkinje cell (*Pden*) cell can also be seen. ×20 000. **c** Higher magnification of the lower molecular layer showing a $P2X_1$-positive neuroglia process interposed between the ascending basket axon (*aBA*) and synaptic dendritic spine (*ds*) (*pf* clusters of parallel fibres, *pfv* varicosity of parallel fibres, *fi* neurofilaments). ×36 000 ▶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ceptors have also been claimed to be present on Purkinje neurons (Kirischuk et al. 1996). P2 receptors have been implicated in glutamate-evoked cytotoxicity in cultured cerebellar granule neurons but the subtype involved has not been investigated (Volonté and Merlo 1996). ATP has also been shown to increase the release of aspartate from cerebellar granule neurons (Merlo and Volonté 1996). 2-Methylthio-ATP has been demonstrated to activate potassium channels in cultured rat cerebellar neurons, the pharmacological data implicating a mechanism via a P2Y receptor subtype (Ikeuchi and Nishizaki 1996).

The existence of both pre- and postsynaptic actions of ATP has previously been suggested based on the immunocytochemistry of $P2X_2$ antisera in the adult rat and guinea-pig cerebellum at the light-microscope level; strong/dense labelling for $P2X_2$ is localized in Purkinje cell soma, dendrites and dendritic spines, neurons in the granular and molecular layers (granule, Golgi, basket and stellate cells), deep cerebellar nuclei, and axons in the white matter (Kanjhan et al. 1996). This is in contrast with the present study of $P2X_1$ receptors where we show that (1) only small populations (minority) of dendritic spines of Purkinje cells and parallel fibres of granule cells display immunoreactivity for $P2X_1$, (2) only immuno-negative varicosities of parallel fibres form synapses with immuno-positive spines and (3) only immuno-positive varicosities are involved in synaptic contacts with immuno-negative dendritic spines. The morphology of the $P2X_1$-labelled fibres indicates that they are parallel fibres but the possibility that there is some labelling of climbing fibres cannot be excluded.

The present results are in contrast to the in situ hybridization and immunohistochemical studies at the light-microscope level; these have reported that no $P2X_1$ mRNA and $P2X_1$ subunits are detected in adult rat brain (Collo et al. 1996; Vulchanova et al. 1996). The discrepancy in the results probably arises because electron microscopy allows more precise observation of the specimens and, in this case, the detection of a small subpopulation of $P2X_1$-positive neuronal profiles of small diameter (parallel fibres, dendritic spines). According to Valera et al. (1994), $P2X_1$ subunits are expressed predominantly in smooth muscle. The prominent localization of $P2X_1$ immunoreactivity in smooth muscle cell membrane is recognized as the postjunctional presence of $P2X_1$ receptor involved in autonomic transmission (Vulchanova et al. 1996). Light microscopy of the localization of $P2X_1$ receptors in the vascular smooth muscle of rat intrarenal vessels (except for efferent arterioles and glomeruli) suggests a paracrine or neurocrine role of extracellular ATP in controlling renal circulation and homeostasis (Chan et al. 1998).

It is well known that the "crossover" synapses of parallel fibres on the spines of Purkinje cell dendrites are the sites of excitatory influences conveyed by granule cells directly to the Purkinje cells (see Paley and Chan-Palay 1974; Ottersen 1993). The highest levels of immunoreactivity to glutamate (fast excitatory neurotransmitter) in the cerebellum have been localized in parallel fibre (and mossy fibre) presynaptic terminals (Somogyi et al. 1986; Clements et al. 1987); immunoreactivity to γ-aminobutyric acid (GABA, mediating inhibition) can be observed in the Purkinje cells (Ottersen and Storm-Mathisen 1984; Ottersen et al. 1988; Reichenberger et al. 1993).

The immunocytochemical data of the present study suggest that purines (ATP) may also act as a neurotransmitter within the intrinsic circuitry of the cerebellum, where $P2X_1$ receptors have been localized postsynaptically (dendritic spines) and presynaptically (parallel fibres). However, the precise physiological significance of $P2X_1$ receptors and the relationship between GABA, glutamate (and other amino acids), nitric oxide (Garthwaite et al. 1988) and purine transmission in the examined region of the cerebellum remains to be determined. According to Lê et al. (1998), ATP may be co-released with excitatory amino acids to control the firing activity of Purkinje cells. Glutamate has previously been shown to act via various classes of receptors, including fast ionotropic (ion-channel-linked) and slower metabotropic (coupled to G proteins) receptors (Batchelor et al. 1994). Brief tetanic stimulation of the glutamate-containing parallel fibre input gives rise to a slow depolarizing synaptic potential that is resistant to ionotropic glutamate receptor blockers and to antagonists acting at GABA receptors (Batchelor and Garthwaite 1993). Interestingly, application of double immunogold labelling to rat cerebellar cortex has revealed that ionotropic receptors (AMPA type) are localized on the membrane opposite the release sites in the main body of the synaptic junction (established by parallel and/or climbing fibre terminals with spines of Purkinje cells), whereas the metabotropic receptors are located at the periphery of the same synapse (Nusser et al. 1994).

Some immunolabelled cell profiles in the rat cerebellum exhibit ultrastructural features typical of neuroglia cells, particularly of astrocytes. The striking finding is that not all the cell, but only certain areas of the cytoplasm, show $P2X_1$ immunoprecipitate. At this stage, it is difficult to associate this finding with any particular function of astrocytes. However, several studies have suggested the involvement of purines in the neuron-glia interaction (Salter and Hicks 1994; Robitaille 1995; Haas et al. 1996; Collo et al. 1997). For example, ATP released from frog motor-end-plates can activate calcium-permeable P2X receptors on perijunctional Schwann cells (Robitaille 1995). Microglia acutely isolated from the brain (corpus callosum) from neonatal mice respond to ATP with an inward current that includes a P2X-like component (Haas et al. 1996). In situ hybridization and imunocytochemical studies suggest the location of $P2X_7$ receptors primarily on microglia (Collo et al. 1997). A pharmacological study of the dorsal spinal cord of the rat indicates that astrocytes express P2 receptors (P2U) that, when stimulated with UTP, produce a rise in the intracellular level of free Ca^{2+} (Ho et al. 1995).

In conclusion, the discrete associations of $P2X_1$ receptors with subpopulations of postsynaptic spines of Purkinje cell dendrites, presynaptic parallel fibres of granule cells and the processes of neuroglia cells (astrocytes) in

rat cerebellum have been revealed at the ultrastructural level. Since other purinoceptors (e.g. $P2X_2$, $P2X_4$) have been observed within the circuitry of the cerebellum (Kanjhan et al. 1996; Lê et al. 1998), the possibility that several subtypes of P2X receptors form heteromultimeric pores cannot be excluded. Interestingly, the co-expression of $P2X_2$ and $P2X_3$ receptors is required to reproduce responses to ATP of sensory neurons (Lewis et al. 1995).

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References

Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475

Batchelor AM, Garthwaite J (1993) Novel synaptic potentials in cerebellar Purkinje cells: probable mediation by metabotropic glutamate receptors. Neuropharmacology 32:11–20

Batchelor AM, Madge DJ, Garthwaite I (1994) Synaptic activation of metabotropic glutamate receptors in the parallel fibre-Purkinje cell pathway in rat cerebellar slices. Neuroscience 63:911–915

Burnstock G (1978) A basis for distinguishing two types of purinergic receptors. In: Straub RW, Bolis L (eds) Cell membrane receptors for drugs and hormones: a multidisciplinary approach. Raven Press, New York, pp 107–118

Burnstock G (1996) Purinergic neurotransmission. Semin Neurosci 8:171–257

Burnstock G (1997) The past, present and future of purine nucleotides as signalling molecules. Neuropharmacology 36:1127–1139

Burnstock G, King BF (1996) Numbering of cloned P2 purinoceptors. Drug Dev Res 38:67–71

Chan CM, Unwin RJ, Oglesby IB, Ford APDW, Townsend-Nicholson A, Burnstock G (1998) Localization of the $P2X_1$ purinoceptors by autoradiography and immunochistochemistry in rat kidneys. Am J Physiol 274:F799-F804

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. Nature 375:428–431

Clements JR, Mongahan PL, Beitz AJ (1987) An ultrastructural description of glutamate-like immunoreactivity in the rat cerebellar cortex. Brain Res 421:343–348

Collo G, North RA, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A, Buell G (1996) Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated channels. J Neurosci 16:2495–2507

Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North RA, Buell G (1997) Tissue distribution of the $P2X_7$ receptor. Neuropharmacology 36:1277–1283

Dunn PM, Blakely AGH (1988) Suramin: a reversible P2-purinoceptor antagonist in the mouse vas deferens. Br J Pharmacol 93:243–245

Edwards FA, Gibb AJ, Colquhoun D (1992) ATP receptor-mediated synaptic currents in the central nervous system. Nature 359: 144–147

Evans RJ, Surprenant A (1992) Vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation is mediated by the release of ATP. Br J Pharmacol 106:242–249

Evans RJ, Derkach V, Surprenant A (1992) ATP mediates fast synaptic transmission in mammalian neurons. Nature 357:503–505

Galligan JJ, Bertrand PP (1994) ATP mediates fast synaptic potentials in enteric neurons. J Neurosci 14:7563–7571

Garthwaite J, Charles SL, Chess-Williams R (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests a role as intercellular messenger in the brain. Nature 336:385–388

Haas S, Brockhaus J, Verkhratsky A, Kettenmann H (1996) ATP-induced membrane current in ameboid microglia acutely isolated from mouse brain slices. Neuroscience 75:257–261

Halliday FC, Gibb AJ (1997) Single-channel properties of P2X ATP receptors in rat cerebellar slices. J Physiol (Lond) 504:51P

Harboe N, Inglid A (1973) Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand J Immunol [Suppl] 1:161–164

Ho C, Hicks J, Salter MW (1995) A novel P2-purinoceptor expressed by a subpopulation of astrocytes from the dorsal spinal cord of the rat. Br J Pharmacol 116:2909–2918

Ikeuchi Y, Nishizaki T (1996) P2 purinoceptor-operated potassium channel in rat cerebellar neurons. Biochem Biophys Res Commun 218:67–71

Kanjhan R, Housley GD, Thorne PR, Christie DL, Palmer DJ, Luo L, Ryan AF (1996) Localization of ATP-gated ion channels in cerebellum using $P2x_2R$ subunit-specific antisera. Neuroreport 7:2665–2669

Kidd EJ, Grahames CB, Simon J, Michel AD, Barnard EA, Humphrey PP (1995) Localization of P2X purinoceptor transcripts in the rat nervous system. Mol Pharmacol 48:569–573

Kirischuk S, Matiash V, Kulik A, Voitenko N, Kostyuk P, Vekhratsky A (1996) Activation of P_2-purino-, α_1-adreno and H_1-histamine receptors triggers cytoplasmic calcium signalling in cerebellar Purkinje neurons. Neuroscience 73:643–647

Lê KT, Villeneuve P, Ramjaun AR, McPherson PS, Beaudet A, Séguela P (1998) Sensory presynaptic and widespread somatodendritic immunolocalization of central ionotropic P2X ATP receptors. Neuroscience 83:177–190

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Heteropolymerization of P2X receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432–435

Maienschein V, Zimmermann H (1996) Immunocytochemical localization of ecto-5'-nucleotidase in cultures of cerebellar granule cells. Neuroscience 70:429–438

Merlo D, Volenté C (1996) Binding and functions of extracellular ATP in cultured cerebellar granule neurons. Biochem Biophys Res Commun 225:907–914

Nori S, Fumagalli L, Bo X, Bogdanov Y, Burnstock G (1998) Co-expression of mRNA for $P2X_1$, $P2X_2$, and $P2X_4$ receptors in rat vascular smooth muscle: an in situ hubridization and RT-PCR study. J Vasc Res 35:179–185

Nusser Z, Mulvihill E, Streit P, Somogyi P (1994) Subsynaptic segregation of metabotropic and ionotropic glutamate receptors as revealed by immunogold localization. Neuroscience 61:421–427

Ottersen OP (1993) Neurotransmitters in the cerebellum. Rev Neurol (Paris) 149:629–636

Ottersen OP, Storm-Mathisen J (1984) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. J Comp Neurol 229:374–392

Ottersen OP, Madsen S, Storm-Mathisen J, Somogyi P, Scopsi L, Larsson LI (1988) Immunocytochemical evidence suggests that taurine is colocalized with GABA in the Purkinje cell terminals, but the stellate cell terminals predominantly contain GABA: a light- and electron-microscopic study of the rat cerebellum. Exp Brain Res 72:407–416

Palay SL, Chan-Palay V (1974) Cerebellar cortex. Cytology and organization. Springer, Berlin Heidelberg New York

Reichenberger I, Streit P, Ottersen OP, Dieringer N (1993) GABA- and glycine-like immunoreactivities in the cerebellum of the frog. Neurosci Lett 154:98–92

Robitaille R (1995) Purinergic receptors and their activation by endogenous purines at perisynaptic glial cells of the frog neuromuscular junction. J Neurosci 15:7121–7131

Salter MW, Hicks JL (1994) ATP-evoked increases in intracellular calcium in neurons and glia from the dorsal spinal cord. J Neurosci 14:1563–1575

Sneddon P, Burnstock G (1984) ATP as a co-transmitter in rat tail artery. Eur J Pharmacol 106:149–152

Sneddon P, Westfall DP (1984) Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea pig vas deferens. J Physiol (Lond) 347:561–580

Somogyi P, Halasy K, Somogyi J, Storm-Mathisen J, Ottersen OP (1986) Quantification of immunogold labelling reveals enrichment of glutamate in mossy and parallel fibre terminals in cat cerebellum. Neuroscience 19:1045–1050

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, Buell G (1994) A new class of ligand-gated ion channel defined by P_{2x} receptor for extracellular ATP. Nature 371:516–519

Volonté C, Merlo D (1996) Selected P2 purinoceptor modulators prevent glutamate-evoked cytotoxicity in cultured cerebellar granule neurons. J Neurosci Res 45:183–193

Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, Elde R (1996) Differential distribution of two ATP-gated ion channels (P_{2x} receptors) determined by immunocytochemistry. Proc Natl Acad Sci USA 93:8063–8067

Vulchanova L, Riedl MS, Shuster SJ, Buell G, Surprenant A, North RA, Elde R (1997) Immunocytochemical study of the $P2X_2$ and $P2X_3$ receptor subunits in rat and monkey sensory neurons and their central terminals. Neuropharmacology 36:1229–1242