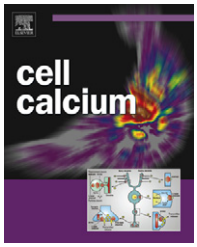
Cell Calcium (2008) xxx, xxx–xxx

journal homepage: www.elsevier.com/locate/ceca



GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx

Roberta Donato[a], Ricardo J. Rodrigues[b], Michiko Takahashi[a], Ming Chi Tsai[a,1], David Soto[a], Kana Miyagi[a], Rosa Gomez Villafuertes[a], Rodrigo A. Cunha[b], Frances A. Edwards[a,*]

[a] *Department of Physiology, University College London, London, UK*
[b] *Center for Neuroscience of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Portugal*

Received 15 June 2007; received in revised form 10 March 2008; accepted 12 March 2008

KEYWORDS
Purkinje cell;
Cerebellar cortex;
Purinergic;
GABA;
GABA release;
Calcium influx;
Calcium stores;
Synaptic transmission;
P2X;
P2Y;
Presynaptic receptors;
IPSC

Summary In many brain regions, Ca^{2+} influx through presynaptic P2X receptors influences GABA release from interneurones. In patch-clamp recordings of Purkinje cells (PCs) in rat cerebellar slices, broad spectrum P2 receptor antagonists, PPADS (30 μM) or suramin (12 μM), result in a decreased amplitude and increased failure rate of minimal evoked GABAergic synaptic currents from basket cells. The effect is mimicked by desensitizing P2X1/3-containing receptors with α,β-methylene ATP. This suggests presynaptic facilitation of GABA release via P2XR-mediated Ca^{2+} influx activated by endogenously released ATP. In contrast, activation of P2Y4 receptors (using UTP, 30 μM, but not P2Y1 or P2Y6 receptor ligands) results in inhibition of GABA release. Immunological studies reveal the presence of most known P2Rs in ≥20% of GABAergic terminals in the cerebellum. P2X3 receptors and P2Y4 receptors occur in approximately 60% and 50% of GABAergic synaptosomes respectively and are localized presynaptically. Previous studies report that PC output is also influenced by postsynaptic purinergic receptors located on both PCs and interneurones.

The high Ca^{2+} permeability of the P2X receptor and the ability of ATP to influence intracellular Ca^{2+} levels via P2Y receptor-mediated intracellular pathways make ATP the ideal transmitter for the multisite bidirectional modulation of the cerebellar cortical neuronal network.

* Corresponding author. Tel.: +44 20 76793286; fax: +44 20 78130530.
E-mail address: f.a.edwards@ucl.ac.uk (F.A. Edwards).
[1] Present address: Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL, USA.

0143-4160/$ – see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.ceca.2008.03.006

Introduction

The ATP-activated P2X receptor/channels (P2XRs) are highly Ca^{2+} permeable. Where purinergic synapses have been recorded in the brain, the amplitude of currents carried by ATP receptor/channels is small and thus it is likely that it is the calcium influx, rather than the depolarization caused by the influx of cations, which is important in their function. This is reflected in the fact that the location of P2XRs has often been found to be presynaptic [1—3] regulating the release of neurotransmitters including the inhibitory transmitter GABA [4—8]. Even where the P2X receptors are found postsynaptically they are thought primarily to regulate such functions as synaptic plasticity because of their calcium permeability [9]. In contrast to P2X receptors, P2Y receptors, including those in Purkinje neurones [10], are usually involved in release of Ca^{2+} from endoplasmic reticulum rather than Ca^{2+} influx. This P2Y-mediated Ca^{2+} release is activated by inositol trisphosphate, via phospholipase C (see [11], for review). Thus, purinergic transmission can in general be considered as a mechanism for regulation of internal Ca^{2+} concentration.

Over recent years purinergic and GABAergic transmission have been repeatedly shown to interact in different brain areas. In some cases ATP and GABA act as co-transmitters, both activating postsynaptic receptors [12]. More commonly however the purinergic receptors are presynaptic [13—15] and can regulate GABA release by altering the background level of calcium in the axon terminal [4—8].

As the entire output of the cerebellar cortex is carried by the firing of Purkinje cells, any mechanism able to modulate the firing pattern of Purkinje cells will influence cerebellar function. Purkinje cells fire spontaneously, even in the absence of glutamate input, and the pattern of firing is very strongly influenced by high conductance, high frequency GABAergic inputs [16]. At least under experimental conditions, this inhibitory input is largely conveyed by two types of inhibitory interneurones, the basket cells and the stellate cells. We have recently demonstrated that, under specific conditions which may prevail during motor activity, the GABA release from the Lugaro cell may also be very important [17].

Considering the importance of GABA transmission in influencing Purkinje cell firing and the many reports that the cerebellum is rich in ATP (P2) receptors [18—21], the interaction between P2 receptors and GABAergic transmission in the cerebellum is clearly of interest. Recently two different groups have approached this question. Saitow et al. [22] reported a role for metabotropic P2Y receptors, demonstrating both a short-term increase in the firing rate of the afferent and a postsynaptic long-term potentiation of GABAergic synaptic activity. In particular, they implicated P2Y receptors in increasing action potential frequency in a population of Lugaro cells but suggested that other GABAergic interneurones may also be recruited. Another group not only showed similar effects of P2Y1 receptors but also implicated P2Y2 and P2Y4 receptors and P2X5 receptors on the soma of basket and stellate cells [23]. The same group later showed that the effects of ATP develop over the first postnatal weeks with the full expression of receptors and effects on release being functional by postnatal day 14 (P14) in rats [24], in agreement with the reported expression of P2X receptor in the cerebellum [25].

Thus, it is clear that, by the end of the second postnatal week, purinergic receptors become important in the control of Purkinje cell function by influencing the firing patterns of interneurones. However, in other brain areas P2 receptors have mostly been found on presynaptic terminals, directly influencing release via Ca^{2+} influx, rather than being located somato-dendritically, controlling the firing of the presynaptic cell (reviewed in [8,13,14]. Thus, in the present study, we specifically investigate the role of P2 receptors on the axon terminals of GABAergic neurones. We use patch-clamp techniques to record synaptic currents in acute brain slices and show that purinergic receptors can modulate GABA release bidirectionally at the level of the GABAergic terminals. By combining pharmacological studies with an immunological characterization of P2 receptors in sub-synaptic fractions and in GABAergic cerebellar terminals, we find that P2X3-containing and P2Y4 receptors are the most likely subtypes involved in the facilitation and inhibition of GABA release, respectively. We go on to investigate how such a complex distribution of P2 receptor-mediated Ca^{2+} modulation, reported here on axons and previously in the soma/dendritic compartments, will affect the firing rates of individual Purkinje cells.

Methods

Electrophysiology

Animals

Male Wistar rats (13—15 days old) were obtained from the University College London animal facility and were killed by decapitation under Home Office License.

Preparation of cerebellar slices

Sagittal cerebellar slices (400 μm thick) were prepared using standard methods [26]. Briefly, the cerebellum was cut along the midline through the vermis and glued on this surface for slicing. Slices were maintained in bubbled Krebs solution at 34 °C for 20—30 min. They were then transferred, within 30 min of slicing, to a second chamber containing fresh bubbled Krebs solution at 34 °C before being allowed to cool to room temperature. All recordings were made at room temperature (21—24 °C) unless otherwise stated.

Solutions. The bath solution (Krebs) contained (in mM): 125 NaCl, 2.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.1 NaH_2PO_4, 25 glucose, and was bubbled with 95% O_2—5% CO_2. In all experiments, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro[f]quinoxaline-7-suphonamide (NBQX; 20 μM) and 7-chlorokynurenate (10 μM) were also included in the bath solution to inhibit ionotropic glutamate receptors. The intracellular solution contained (in mM): 140 CsCl; 5 HEPES; 10 EGTA; 2 MgATP; and 2 $CaCl_2$; pH 7.4 with CsOH.

NBQX was dissolved in DMSO at 500× its final concentration. Aliquots of the concentrated NBQX were frozen at −20 °C and diluted into Krebs solution at the time of the experiment. 7-chlorokynurenate was dissolved in NaOH at

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

500× its final concentration (pH adjusted to 7.4). All other drugs were dissolved in distilled water, and concentrated aliquots were similarly frozen.

Recording synaptic currents

Thick-walled borosilicate glass electrodes (World Precision Instruments, Hertfordshire, UK) were pulled to a tip resistance of ~4 MΩ for recordings from Purkinje cells (PP-83 microelectrode puller; Narishige, Tokyo, Japan). Purkinje cells were visually identified under infrared differential interference contrast microscopy, using a CCD camera (Hitachi, model KP-M1E/K) mounted on an upright microscope (BX50WI; Olympus Optical, Tokyo, Japan). Whole-cell voltage-clamp recordings were made from the somata of Purkinje cells with an Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, USA), using standard methods [26]. The membrane potential was held at −70 mV resulting in inward Cl^- currents with the intracellular solution used. Pipette series resistance was typically 10–20 MΩ. Using a test pulse of −10 mV, 50 ms, the input resistance, series resistance and capacitance were monitored throughout. Series resistance and capacitance were not compensated but the recording was abandoned if the series resistance changed by more than 15%. The CsCl-based intracellular solutions have negligible junction potentials (approximately 1 mV).

Evoked synaptic currents. Synaptic currents were evoked using minimal stimulation by applying bipolar rectangular voltage pulses (100–200 μs, 0.5 Hz; Grass SD9 stimulator; Astro-Med, West Warwick, USA). Stimulating electrodes were pulled in the same way as recording electrodes but filled with 1 M NaCl. The tip of the stimulating electrode was placed in the molecular layer approximately 100 μm away from the recorded PC and about 30–50 μm above the PC layer. The stimulating voltage increased from zero until a synaptic current was observed that had an all-or-none relationship to stimulus strength. Currents were recorded from the amplifier at a bandwidth of 10 kHz (four pole Bessel) and usually sampled on-line through a 2 kHz filter (eight pole Bessel; Frequency Devices, Haverhill, USA) at a sampling frequency of 10 kHz with an Axon Digidata 1200 (Axon Instruments) using the program WinWCP (kindly supplied by Dr. J. Dempster, University of Strathclyde, Glasgow, UK; available at http://www.strath.ac.uk/Departments/PhysPharm/ses.htm). Note that for suramin experiments minimal stimulation was not used for all cells. In these experiments stimulus strength was set to obtain 5–10% failures in control recordings.

Miniature synaptic currents. Miniature currents were recorded similarly to above but without stimulation. Tetrodotoxin (1 μM) was included in the bath solution to prevent sodium action potentials. Currents were recorded continuously onto tape and WinEDR (supplied by John Dempster as above) was used to detect the currents that remained below a threshold of −5 pA for at least 5 ms, with a dead time between consecutive events of 15 ms. This results in an effective detection threshold of −8 to −12 pA. Note that as outlined previously, the miniature inhibitory synaptic currents in Purkinje cells are very large under these recording conditions (so that the smallest detected miniature current was 37 pA, giving a very high signal to noise ratio).

Immunohistochemistry

Animals

Male Wistar rats (16–18 days old) were obtained from Harlan Ibérica (Barcelona, Spain) and were handled according with the EU guidelines for use of experimental animals, the rats being anesthetized under halothane before being sacrificed by decapitation.

Sub-synaptic distribution of P2 receptors in the rat cerebellum

The solubilization of non-active zone, presynaptic active zone and postsynaptic fractions from rat cerebellar synaptosomes was performed according to the method previously described [27], with minor modifications [28]. Briefly, the cerebella from 8 male Wistar rats were homogenized at 4 °C with a Teflon–glass homogenizer in 15 ml of isolation solution (0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, PMSF). The concentration of sucrose was raised to 1.25 M by addition of 75 ml of sucrose (2.0 M) and 30 ml of $CaCl_2$ (0.1 mM) and the suspension divided into 10 ultracentrifuge tubes. The homogenate was overlaid with 8 ml of sucrose (1.0 M), 0.1 mM $CaCl_2$, and with 5 ml of homogenization solution and centrifuged at 100,000 × *g* for 3 h at 4 °C. Synaptosomes were collected at the 1.25/1.0 M sucrose interface, diluted 1:10 in cold 0.32 M sucrose with 0.1 mM $CaCl_2$ and pelleted (15,000 × *g*; 30 min; 4 °C). Pellets were resuspended in 1 ml of 0.32 M sucrose with 0.1 mM $CaCl_2$ and a small sample taken for gel electrophoresis. The synaptosomes were then solubilized to obtain the sub-synaptic fractions, as previously described [27,28]. Briefly, the synaptosomal suspension was diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2X solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) pelleted (40,000 × *g*; 30 min; 4 °C). The supernatant (extrasynaptic fraction) was decanted and proteins precipitated with 6 volumes of acetone at 20 °C and recovered by centrifugation (18,000 × *g*; 30 min; 15 °C). The synaptic junctions pellet was washed in pH 6.0 solubilization buffer, resuspended in 10 ml of 1% Triton X-100 and 20 mM Tris, pH 8.0, incubated for 30 min on ice with mild agitation, centrifuged (40,000 × *g*; 30 min; 4 °C) and the supernatant (presynaptic fraction) processed as above. PMSF (1 mM) was added to the suspension in all extraction steps. The pellets from the supernatants and the final insoluble pellet (postsynaptic fraction) were solubilized in 5% sodium dodecyl sulfate (SDS), the protein concentration determined by the bicinchoninic acid protein assay and the samples were added to a 1/6 volume of 6× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer prior to freezing at 20 °C.

Western blot analysis was carried out as previously described [28] using 30–80 μg of each protein fraction, obtained as described above, which were loaded onto 7.5% SDS-PAGE gel and then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h at room temperature in 5% low-fat milk in Tris buffered saline medium with 0.1% Tween 20 (Sigma). The membranes were probed with primary antibodies raised against

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

P2X1 (1:500), P2X2 (1:500), P2X3 (1:200), P2X4 (1:500), P2X5 (1:200), P2X6 (1:200), P2X7 (1:15,000), P2Y1 (1:200), P2Y2 (1:500), P2Y4 (1:500), P2Y6 (1:200) or P2Y11 (1:500), applied overnight at 4 °C. The detection was performed using alkaline phosphatase conjugated secondary antibodies goat anti-rabbit IgG (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK; 1:20,000) or rabbit anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA; 1:5,000). Immunoblots were visualized using Amersham's ECF detection reagent and quantified using a VersaDoc3000 quantitative digitiser (Biorad). Note that the specificity of all antibodies has previously been tested and the appropriate conditions established [28].

Localization of P2 receptors immunoreactivity in GABAergic nerve terminals of the rat cerebellum

For immunochemical analysis, cerebellar synaptosomes were obtained through a discontinuous Percoll gradient, following the procedure described by Díaz-Hérnández et al., [29], with minor modifications [28]. The cerebella were homogenized in a medium containing 0.25 M sucrose and 5 mM TES (pH 7.4). The homogenate was spun for 3 min, 2000 × *g* at 4 °C and the supernatant spun again at 9500 × *g* for 13 min. The pellets were then resuspended in 8 ml of 0.25 M sucrose and 5 mM TES (pH 7.4). Then, 2 ml of this synaptosomal suspension were placed onto 3 ml of Percoll discontinuous gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol and 3, 10 or 23% Percoll, pH 7.4. The gradients were centrifuged at 25,000 × *g* for 11 min at 4 °C. Synaptosomes were collected between the 10% and 23% Percoll bands and diluted in 15 ml of HEPES buffered medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). After centrifugation at 22,000 × *g* for 11 min at 4 °C, the synaptosomal pellet was removed.

Rat cerebellar synaptosomes were placed on coverslips previously coated with poly-L-lysine, fixed with 4% paraformaldehyde for 15 min and washed twice with PBS medium (140 mM NaCl, 3 mM KCl, 20 mM NaH_2PO_4, 15 mM KH_2PO_4, pH 7.4). A permeabilization step was performed in PBS containing 0.2% Triton X-100 for 10 min and then the synaptosomes were incubated in PBS medium containing 3% bovine serum albumin (BSA) and 5% normal rat serum for 1 h. The synaptosomes were then washed twice with PBS medium and incubated with primary antibodies raised against P2X1 (1:500), P2X2 (1:500), P2X3 (1:400), P2X4 (1:500), P2X5 (1:400), P2X6 (1:400), P2X7 (1:15,000), P2Y1 (1:400), P2Y2 (1:500), P2Y4 (1:500), P2Y6 (1:400) or P2Y11 (1:500) together with guinea-pig anti-vGAT (1:1000) and mouse anti-synaptophysin for 1 h at room temperature. The synaptosomes were then washed three times with PBS with 3% BSA and were incubated for 1 h at room temperature with AlexaFluor-488 (green) labelled donkey anti-goat or chicken anti-rabbit IgG antibodies and AlexaFluor-598 (red) labelled goat anti-guinea pig and AlexaFluor-350 (blue) labelled goat anti-mouse (1:200 for all). After washing and mounting on slides with Prolong Antifade (Molecular Probes), the preparations were visualized in a Zeiss Axiovert 200 (Germany) inverted microscope equipped with a cooled CCD camera and analyzed with MetaFluor 4.0 software.

Antibodies

The primary antibodies against P2X1, P2X2, P2X4, P2X7, P2Y2, P2Y4 were from Alomone labs (Israel), against P2X3, P2X5, P2X6, P2Y1, P2Y6 were from Santa Cruz biotechnology (Santa Cruz, USA), against P2Y11 was from Zymed (Lisboa, Portugal), the antibody against vGAT was from Calbiochem (Darmstadt, Germany) and the antibody against synaptophysin was from Sigma (Sintra, Portugal).

Statistics

Results are presented as mean ± S.E.M. and statistical differences between means were considered significant if the probability of chance occurrence was ≤0.05 using the Student's *t*-test.

Results

Endogenous ATP inhibits the release of GABA from basket cells and possibly stellate cells through P2 receptor activation

Membrane currents were recorded in Purkinje cells using whole-cell patch-clamp techniques in parasagittal cerebella slices in the presence of glutamate receptor antagonists (NBQX, 20 μM and 7Cl-kynurenate, 10 μM). $GABA_A$ receptor-mediated inhibitory synaptic currents (IPSCs) were evoked extracellularly using standard methods for stimulating the axons of the common cerebellar interneurone, the basket cell (Fig. 1A as outlined in 'Methods'). The evoked currents and the background spontaneous activity could be completely and reversibly blocked by addition of the $GABA_A$ receptor antagonist SR95531 (6 μM, Fig. 1B).

When the broad spectrum antagonist of P2 receptors, PPADS (30 μM), was added to the bath solution, the mean amplitude of the minimal evoked IPSCs was reversibly reduced by about 25% ($p < 0.05$; Fig. 1Cii and D). The proportion of stimuli that resulted in a failure to record a synaptic current was significantly increased ($p < 0.05$; Fig. 1Ci and E). The increase in failures strongly suggests that the inhibition caused by P2 receptor antagonists is largely due to a presynaptic inhibition of release of GABA, rather than an effect on the postsynaptic $GABA_A$ receptors. Similar effects were seen with another broad spectrum P2 receptor antagonist suramin (12 μM; $n = 8$; amplitude decreased to 39 ± 7% $p < 0.01$; failures increased from 6% to 23%, $p < 0.05$. Note that as outlined in 'Methods' the suramin stimulation was not minimal and hence the failure rate was lower than seen in the minimal stimulation experiments for PPADS application.). In order to confirm that the tonic P2 receptor-mediated facilitation of GABAergic transmission was directly exerted at the presynaptic level by controlling the vesicular release of GABA, we went on to observe the effect of blocking P2 receptors on miniature inhibitory synaptic currents.

In the presence of tetrodotoxin (1 μM) and glutamate antagonists, as above, Purkinje cells still feature a high frequency of large synaptic currents, which were completely blocked by $GABA_A$ receptor antagonists (Fig. 2A). In the presence of PPADS (30 μM), the frequency of these miniature synaptic currents decreased by approximately 30% (Fig. 2B and C) with a smaller (14%) but significant

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

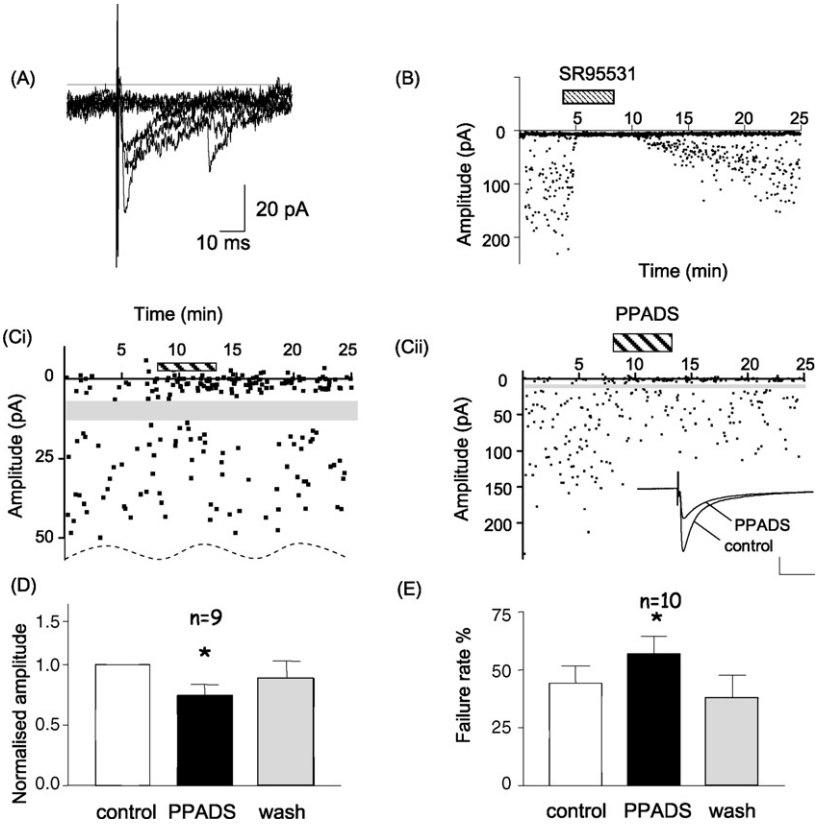


Figure 1 Evoked GABA currents in the cerebellar cortex are blocked by $GABA_A$ receptor antagonist, SR95531 and partially inhibited by the non-selective P2 antagonist, PPADS. (A) Typical evoked GABAergic currents in the cerebellar cortex. Evoked currents are lined up by the stimulus artifact at the beginning of each trace. Spontaneously occurring currents can also be observed occurring randomly between stimuli. The darker trace is the average of 6 evoked currents. (B) The effect of bath application of SR95531 (6 μM) on peak amplitudes over time of evoked GABAergic synaptic currents in a typical cell. The bar above represents the time course of drug application. This and all experiments were carried out in the presence of antagonists of ionotropic glutamate receptors (see 'Methods'). (C) The effect of bath application of PPADS (30 μM, 5 min) on peak amplitudes over time of evoked GABAergic synaptic currents in a typical cell. The striped bar above represents the time course of PPADS application. (Ci) Only currents ≤50 pA in order to emphasize the clear gap between failures and currents (grey bar). (Cii) The same data as shown in Ci but with the full amplitude range. Note that not only do amplitudes appear smaller but the density of failures is increased. *Inset*: Averaged evoked currents before and after application of PPADS. Scale bar 50 pA, 50 ms. (D) Summary of mean GABAergic-evoked current amplitudes before, during application and after washout of PPADS. (E) Summary of failure rates before, during application and after washout of PPADS.

change in amplitude (Fig. 2D). Both effects reversed on washing back to control solution. This confirms the presynaptic effect of PPADS but suggests that there may also be a small postsynaptic contribution. However, as the study is largely concentrating on presynaptic effects, 10 mM EGTA is included in the intracellular solution, which would largely prevent any Ca^{2+}-dependent postsynaptic effects. It is possible that the small effect seen reflects a calcium-independent effect or a Ca^{2+}-dependent effect due to influx through a P2X receptor which was too fast to be buffered by EGTA. We did not however pursue this effect further in this study. Note that there was no measurable change in input resistance (Fig. 2E). However, as Purkinje cells have a very extensive dendritic tree, even at P14, it is likely that small local changes in the dendrites, which might affect the cable properties of the cell, would not be detectable at the soma. As the amplitude of the miniature synaptic currents in Purkinje cells, with or without PPADS, is well above threshold for detection, the detected frequency of synaptic currents would not be affected by small changes in amplitude. Thus, it is possible to conclude that the blockade of P2 receptors by PPADS results in inhibition of the release of GABA. This is most likely due to blockade of presynaptic P2 receptors activated by endogenous nucleotides, which tonically enhance GABAergic transmission, mainly by increasing release of GABA. Some postsynaptic effects of tonic nucleotide release may also be present.

P2X1- or P2X3-containing receptors control GABA release

As PPADS and suramin block most P2X and P2Y receptors except for P2X4 and P2X6, we investigated the effects of some of the more subtype-specific compounds. One of the few compounds which are specific for P2X receptors over P2Y receptors is α,β-methylene ATP which is an agonist selective for P2X1 and P2X3 containing receptors [30]. These receptors desensitise rapidly and for long periods and

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

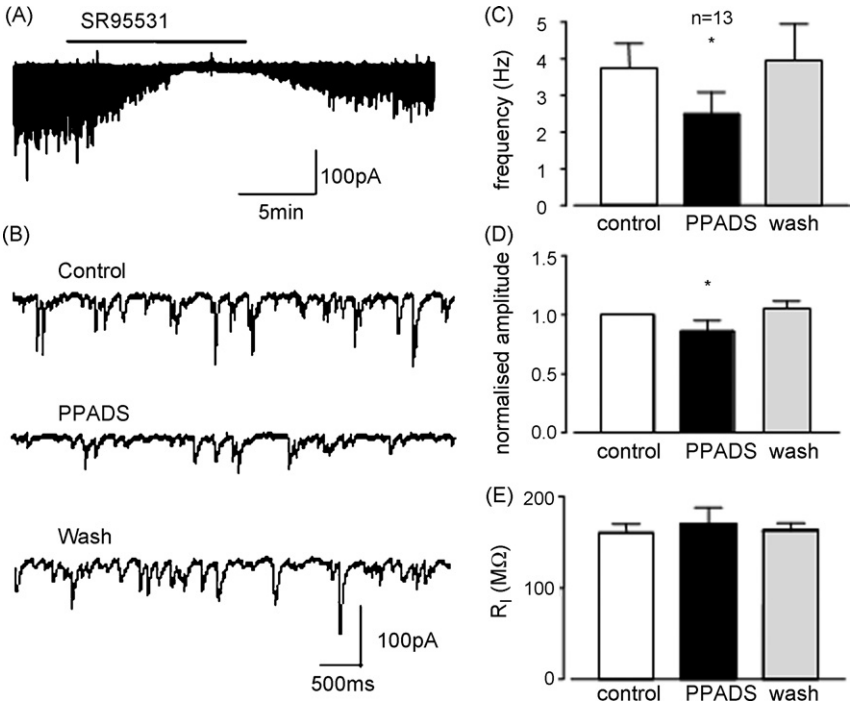


Figure 2 Effect of the broad spectrum P2 receptor antagonist PPADS (30 μM) on miniature GABAergic synaptic currents. (A) Miniature currents (in TTX 1 μM, NBQX, 20 μM and 7-Cl kynurenate 5 μM) were completely blocked in the presence of SR95531 (6 μM) confirming them to be GABAergic. (B) Raw data showing inhibition of both amplitude and frequency of GABAergic miniature currents in the presence of PPADS. (C–E) Summary of pooled data showing the effects of PPADS on C, frequency and D, amplitude of miniature synaptic currents and E, the lack of effect on PC input resistance.

hence addition of α,β-methylene ATP to the bath solution will effectively inactivate such receptors and so would be expected to have a similar effect to an antagonist. Under the present conditions, α,β-methylene ATP (20 μM) has a similar effect to PPADS. Although the apparent decrease in amplitude (~30%) did not reach significance, the trend was in the same direction and the failure rate of evoked IPSCs was significantly increased ($p < 0.01$; Fig. 3A), suggesting a presynaptic role for P2X1 or 3 containing receptors. Note that, in contrast to PPADS (which competitively blocks P2 receptors, allowing reversal of the effect on washout), the desensitization of P2X1/3 receptors by α,β-methylene ATP is long-lasting and does not allow the reversal of the effect on washout.

P2Y receptors may also influence GABA release

MRS2179 is a selective antagonist of P2Y1 receptors [31]. Addition of MRS2179 (1 or 10 μM) has no measurable effect on evoked IPSCs under the same conditions as above (Fig. 3C). Similarly UDP (30 μM), which is an agonist of P2Y6 receptors, has no effect (Fig. 3D). In contrast, UTP (30 μM), which is an agonist of P2Y4 and P2Y2 receptors, increases the failure rate significantly ($p < 0.05$) without causing a significant change in the amplitude of the post-synaptic currents ($p > 0.05$; Fig. 3B). As the metabotropic P2Y receptors are not expected to desensitise to any substantial extent under these conditions, this inhibitory effect of UTP suggests that activation of P2Y receptors inhibits GABA release thus having the opposite effect to activation of P2X receptors. Overall however the effect of endogenously released ATP seems to be P2X-mediated facilitation as revealed by blocking both receptor types. Moreover as this effect is clearly not chronically desensitized it must relate to rapidly released and removed ATP rather than ATP which is permanently present within the extracellular environment.

Identification of the P2 receptors located in the active zone of cerebellar nerve terminals

All the above evidence strongly suggests that both P2X and P2Y receptors should be present in or near to the presynaptic active zone of GABAergic nerve terminals in the cerebellar cortex. We thus attempted to verify their presynaptic location using a recently described technique that allows the separation of fractions enriched for presynaptic active zone proteins, postsynaptic proteins and presynaptic proteins from outside the active zone (extra-synaptic fraction) obtained from solubilized nerve terminals [27]. As previously validated for other brain areas [28], we confirmed that this separation in the cerebellum was over 90% effective as gauged by Western blot analysis of syntaxin (presynaptic active zone marker), PSD95 (postsynaptic active zone marker) and synaptophysin (presynaptic terminal marker not associated with the active zone) (Fig. 4A). When immunoreactivity was tested with a variety of antibodies to different P2 subunits, previously validated as

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

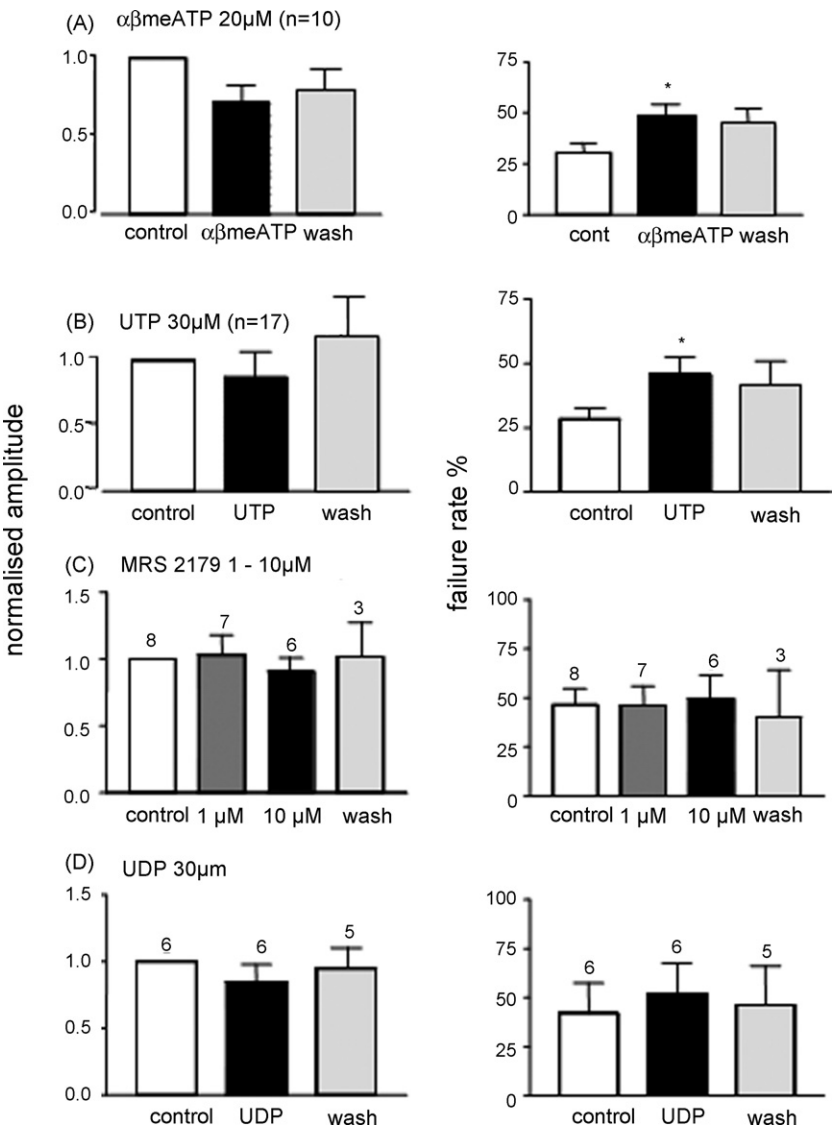


Figure 3 Effect of selective antagonists and agonists of P2 receptors on evoked GABAergic synaptic currents. The left column summarizes the effects on amplitude (normalized to control) and the right column shows the effect on failures of (A) α,β-methylene ATP, the desensitizing agonist of P2X1 or P2X3 containing receptors; (B) UTP, P2Y4 (and in some cases P2Y2) receptor agonist; (C) MRS2179, the P2Y1 receptor antagonist and (D) UDP, the P2Y6 receptor agonist.

selective for each of the different P2 receptor subtypes [28], a differential staining was observed in the different fractions for each of the tested P2 receptors. As illustrated in Fig. 4B, it is clear that the most abundant P2X receptors in the presynaptic active zone are P2X2 and P2X3 receptors. Both these receptors also occur postsynaptically but less abundantly (proportion of total immunoreactivity occurring presynaptically: P2X2, 58.1 ± 1.2%, $n = 3$; P2X3, 55.6 ± 1.5%, $n = 3$). P2X5 and P2X6 receptors, although less abundant than P2X2 and 3 are also more abundant pre than postsynaptically (P2X5, 59.1 ± 1.8% presynaptic, $n = 3$; P2X6 receptors, 96.6 ± 1.1%, $n = 3$)). As for P2Y receptors, only P2Y4 and P2Y6 are present to a significant extent in the presynaptic active zone (37.3 ± 1.6% and 52.1 ± 2.1% of the total immunoreactivity, respectively, $n = 3$), with P2Y1, P2Y2 and P2Y4 antibodies predominantly labelling the postsynaptic fraction (Fig. 4B).

Identification of the P2 receptors located in GABAergic terminals of the cerebellum

Since the fractionation procedure used above does not allow the purification of only the sub-synaptic fractions from GABAergic terminals, we undertook a complementary double immunocytochemistry study in single nerve terminals aimed to identify the P2X and P2Y receptors located in particular in cerebellar GABAergic synaptosomes. Thus, we quantified to what extent the immunoreactivity of the different P2 receptors was co-localized with vesicular GABA transporters (VGAT) immunoreactivity, a label for GABAergic nerve terminal (see Fig. 5). As presented in Fig. 5B, all subtypes of the P2 receptors tested appear to be present on some GABAergic synaptosomes, but the P2X receptor subunits located in the greatest proportion of GABAergic terminals were P2X3, P2X5 and P2X6. As for the P2Y

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

receptors, more than 70% of GABAergic synaptosomes were positive for P2Y1 receptors with P2Y4 and P2Y6 appearing in around 50%.

Note that synaptosomes such as those used here will contain both the terminal and the postsynaptic density. Comparing this double immunocytochemistry data with the data obtained in the sub-synaptic fractionation of the presynaptic active zone, it seems that the P2X3 and P2Y4 receptors implicated in the electrophysiological experiments are both abundantly located in GABAergic synaptosomes and are preferentially located in the presynaptic active zone.

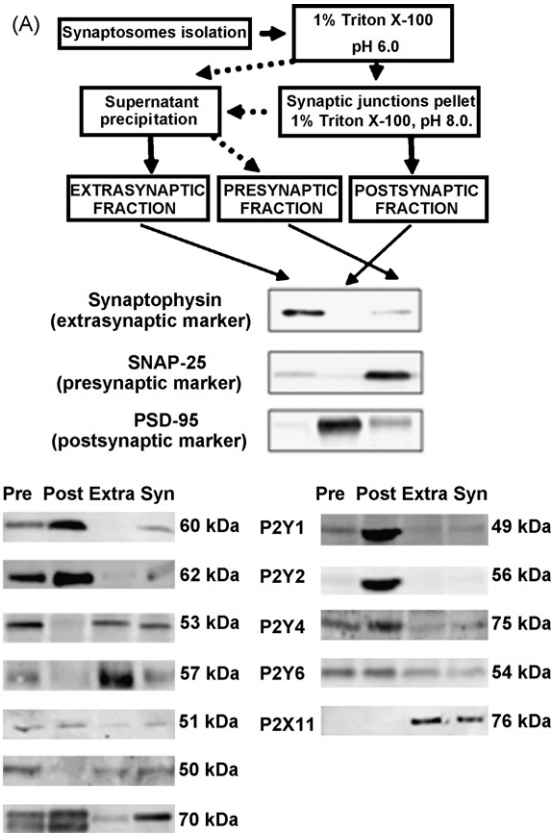


Figure 4 Sub-synaptic distribution of P2X and P2Y receptors in the cerebellum. (A) Selective antibodies for each P2X and P2Y receptor were tested by Western blot analysis in a fraction enriched in the presynaptic active zone (pre), in the postsynaptic density (post), in nerve terminals outside the active zone (extra) and in the initial synaptosomal fraction (syn) from which fractionation began. These fractions were over 90% pure, as illustrated by the ability to recover the immunoreactivity for SNAP25 in the presynaptic active zone fraction, PSD95 in the postsynaptic density fraction and synaptophysin (a protein located in synaptic vesicles) in the extrasynaptic fraction. (B) Western blots of these fractions evaluating the sub-synaptic distribution of the immunoreactivity of the antibodies selective for each P2X subunit and each P2Y receptor tested (20—80 μg of protein of each fraction were applied to SDS-PAGE gels). Each blot is representative of at least 3 blots from different groups of animals with similar results. For each fractionation procedure, Western blot analysis for the markers of each fraction was performed as illustrated in (A), in order to assess the efficiency of each fractionation.

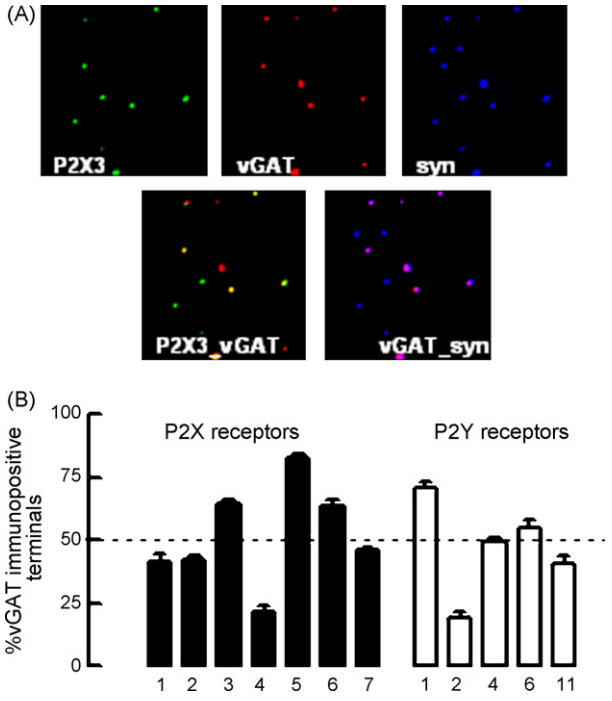


Figure 5 Identification of P2X and P2Y receptors present in GABAergic boutons by triple immunocytochemical analysis of rat cerebellar single nerve terminals. (A) An example of the immunocytochemical identification of P2X3 receptors subunit (left panel, first row), GABAergic nerve terminals identified as immunopositive for vesicular GABA transporters (central panel, first row), and the total synaptosomal population identified as immunopositive for the synaptic marker synaptophysin (right panel, first row). The proportion of GABAergic terminals was measured after merging vGAT, and synaptophysin (syn) images (right panel, second row) comprised $54.2 \pm 3.1\%$ ($n = 4$). Merging images of vGAT and P2 antibody labelling (left panel, second row) allowed the percentage of rat cerebellar GABAergic nerve terminals endowed with each P2X subunit and P2Y receptor to be identified. (B) Summary of all the proportion of GABAergic terminals found to be positive for each of the subunits tested. The data are mean ± S.E.M. of 3—4 experiments and in each experiment, using different synaptosomal preparation from different animals, four different fields acquired from two different coverslips were analyzed.

The firing patterns of Purkinje cells

It thus seems clear that activation of different presynaptic purinergic receptors by background ATP causes a basal increase in GABA release (seen as inhibition of release when both receptors are blocked by PPADS). However, is this effect on GABA release important in terms of the output of the cerebellum? In the light of the many sites and types of action of P2 receptors in this circuitry, it is not possible in these experiments to test the role of any particular site of purinergic modulation on the end effect of Purkinje cell firing. However, we have observed that, under the basal conditions of slice physiology, presynap-

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

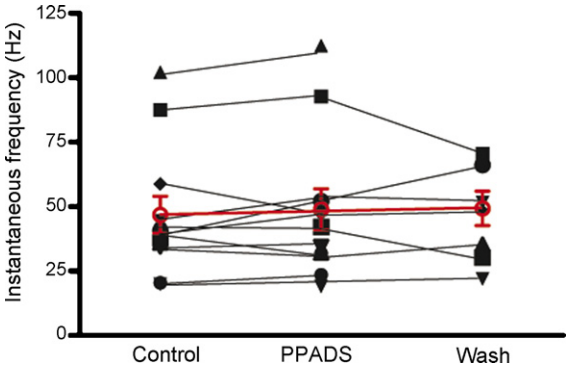


Figure 6 Effect of bath application of PPADS on Purkinje cell firing. A scatter plot of data from cell-attached recordings from individual cells shows that bath application of PPADS (30 μM, 34 °C) caused a range of effects on instantaneous firing rates (black symbols). There was however no mean change across the cells tested (red symbols, mean ± S.E., $n = 12$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tic P2X receptors dominate over P2Y receptors in terms of GABA release from basket cells. We thus investigated whether any one of the many observed effects of ATP-mediated receptor modulation dominates in the end effect of the firing of Purkinje cells. To this end we observed the effect of blocking all PPADS-sensitive receptors in the network by recording the spontaneous rate of firing of action potential in Purkinje cells in cell-attached mode and measuring the effect of PPADS on their spontaneous firing rates both at room temperature (data not shown) and at 34 °C (Fig. 6). While small significant increases or decreases were seen in individual neurones, on average PPADS had no significant effect on firing rate either under conditions of tonic or bursting activity. A small but significant inhibitory effect on Purkinje cell firing (10% decrease, $n = 10$, $p < 0.05$) at approximately physiological temperatures (34 °C) was observed but only in the presence of glutamate antagonists.

Discussion

The primary finding in this paper is the demonstration of direct bidirectional presynaptic modulation by P2 receptors of GABA release onto Purkinje cells in cerebellar slices. Activation of P2X receptors (probably $P2X_3$-containing) facilitates GABA release from basket cells and possibly stellate cells while activation of $P2Y_4$ receptors inhibits it.

The present data add to the previous observations that activation of P2 receptors increases the firing of all classes of cerebellar interneurones [22,23]. Brockhaus et al. also showed that the P2X receptors enhanced the GABAergic transmission in cerebellar slices [23]. However, rather than direct modulation of GABA release, they reported that activation of P2X receptors enhances the firing rate of GABAergic neurons. On the other hand, Saitow et al. [22] specifically patched the Lugaro cell showing that a P2Y receptor agonist caused firing of this particular type of interneurone without affecting the vesicular release of GABA from these cells directly [22]. In the present study, we focused on presynaptic P2X receptors and demonstrate that P2X receptors can control GABA release directly at the level of the boutons impinging on Purkinje cells. Although we have not directly identified the interneurones stimulated, it is unlikely that the Lugaro cells substantially contribute to our results. We have previously studied Lugaro cells under these conditions [17] and have established that the placement of the stimulating electrode in the present study would be unlikely to activate a Lugaro cell. Instead, we would certainly activate basket cells and it is possible that stellate cells are also in some cases in range. Furthermore, our observations on acute and reversible effects of P2 receptor activation on GABAergic transmission are likely to be complimentary to the observation of Saitow et al. who found long-term plastic changes operated by P2Y1 receptors [22]. While we were unable to detect any effect of the P2Y1 receptor antagonist MRS2179, these results are not incompatible as our conditions were different to those of Saitow et al. They detected this effect in response to a P2Y agonist and showed it to be prevented by the P2Y1 receptor antagonist. In fact it would not be possible to detect such a long-term change without application of an agonist, as the antagonist would have to be present at the time of receptor activation. If long-term potentiation had already occurred as a result of endogenous ATP, application of the antagonist would not be expected to reverse it. Moreover by including 10 mM EGTA in all our intracellular solutions we have specifically prevented most Ca^{2+}-dependent postsynaptic effects. Thus, the effects on GABA release observed here are probably additional to the various other interactions of P2 receptors with the GABAergic neurones previously reported in the cerebellum.

One of the novel findings of our study is the presence of a dual and opposite modulation via P2 receptors of GABAergic transmission. In fact, our results indicate the presence of a tonic facilitation operated by P2X receptors, together with an inhibitory effect resulting from P2Y receptor activation which is uncovered when a P2Y agonist is added to the bath. Furthermore we have confirmed that both P2X and P2Y receptors are present in the presynaptic active zone and in GABAergic terminals of the cerebellum, as evaluated by immunocytochemistry and Western blot analysis. This provides the first demonstration in the cerebellum of the simultaneous presence of both P2X and P2Y receptors with opposing effects on the same pathway (transmitter release), as was previously shown to occur in glutamatergic synapses of the rat medial habenula nucleus [32] and in glutamatergic terminals of the rat hippocampus [28]. Interestingly, we found that dominant effect of tonic endogenous P2 receptors activation (presumably by constantly released ATP) was enhancement of GABA release via the P2X receptor. In fact, the generic P2 receptor antagonists, PPADS or suramin, caused an inhibitory effect similar in amplitude to that caused by the selective desensitization of P2X3-containing receptors with α,β-methylene ATP. This suggests that P2Y receptors have little if any influence under these conditions. Indeed, previous studies in hippocampal synapses [28], as well as in the noradrenergic varicosities of the vas deferens [33], also showed a prevalence of P2X receptor-mediated facilitation over P2Y receptor-mediated inhibition of neurotransmitter release with increasing lev-

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

els of adenine nucleotides. Considering that P2X receptors have a lower affinity for ATP than P2Y receptors, this may suggest a specific localization of the two receptor subtypes compared to the release site of ATP. However, such separation may not be necessary. Ca^{2+} influx is the final rate-limiting step in synaptic transmission and thus decreasing the importance of the voltage-gated Ca^{2+} channels by allowing Ca^{2+} influx via P2X receptors may bypass any intracellularly mediated effects of P2Y receptors on transmitter release.

One key issue which would be of interest in future studies is the source of the endogenous ATP which is tonically activating the P2X receptors in cerebellar GABAergic synapses. ATP can be vesicularly co-released with different neurotransmitters (see [13]), especially with GABA [12,34]. This would be expected to yield high transient concentrations in the synaptic cleft that are likely to reach levels sufficient to activate the lower affinity P2X receptors. Indeed, the release of ATP from cerebellar nerve terminals was shown to be greater than other tested brain areas [35]. Alternatively, ATP can be released from astrocytes (reviewed in [36,37]) resulting in volume transmission which is expected to lead to lower concentrations of ATP in synapses that might activate the higher affinity P2Y receptors selectively [38]. Interestingly, in slice studies focusing on circuit modulation, it is mostly observed that astrocytic-derived ATP is involved in heterosynaptic depression [39–41].

The combination of functional pharmacology with biochemistry and histochemical immunology allowed us to suggest a molecular characterization of the P2 receptors involved in this dual inhibition and facilitation of GABA release from cerebellar synapses. In agreement with previous studies, we confirmed the importance and functional relevance of the P2X3 receptor subunit [21,42]. We were specifically able to confirm the presence of P2X3 receptors in GABAergic terminals. Moreover, considering their prevalence, we suggest that P2X5 and P2X6 subunits may form heteromers with P2X3 receptors. The isolation of GABAergic terminals was important here as they take up only about 2% of total volume in brain tissue (see e.g. [43,44]) which make this compartment difficult to evaluate histologically using slice tissue. This is probably the reason for the inability in some studies to detect the presence of P2X3 receptors in the cerebellum [25]. Note that alone the immunohistochemical experiments cannot be very conclusive because there are many types of GABAergic neurones in the cerebellar cortex and indeed the basket cell terminals onto Purkinje cells may not be the most abundant of these. However, these data agree closely with the pharmacology results and thus further support the conclusions of this section of the study.

With respect to the P2Y receptors located in GABAergic terminals in the cerebellum, we mostly detected the presence of P2Y4 and P2Y6, although the pharmacological studies only revealed a role of the former in GABA release. Interestingly, the sub-synaptic fractionation revealed that all subtypes of P2Y receptors were most abundant in the postsynaptic density. This is compatible with our report of a small but significant effect of PPADS on miniature current amplitude and that of Saitow et al. on postsynaptic effects of P2Y1 receptors in Purkinje cells [22]. It is also compatible with previous reports of the localization of abundant P2Y receptors in cerebellar neurons of the molecular layer [45–47]. However, we did not follow up this result further as we were primarily interested in presynaptic effects. In this respect, it is should be noted that others have also provided evidence for functional P2X receptors in neonatal Purkinje cells (e.g. [25,48]).

Taking together the results of the present study and past reports, it is clear that a wide variety of different P2 receptors are involved in the control of transmission in different loci in neuronal network which makes up the cerebellar cortex. ATP, which largely acts by controlling Ca^{2+} influx into presynaptic terminals and postsynaptic compartments is thus an ideal transmitter to exert subtle control on all levels of the circuit. Even if we restrict our considerations to the inhibitory neurones, it seems that purinergic receptors control GABAergic transmission onto Purkinje cells at several different levels. Thus, depending on where it is released ATP will cause both a change in the firing patterns of different interneurones and in the case of basket cells a change at the level of the presynaptic terminal. The various roles of purinergic transmission in controlling Purkinje cell function are summarized in Fig. 7.

The question arises as to what the end effect of these complex interactions will be on Purkinje cell firing. In the brain slice the circuitry is limited and clearly the control of all the individual elements under conditions of motor activity cannot be tested. However, in an attempt to assess whether any purinergic locus dominated, the effect on PC firing of a wide ranging block of P2 receptors was observed. This blanket block of purinergic receptors resulted in minor increases or decreases in individual neurones but no mean population effect on the rate of Purkinje cell firing, unless the circuit was further limited by blocking glutamatergic transmission. Such a non-specific block of most purinergic receptors in the slice is however clearly not physiological and may have very different effects from what would be observed if the different parts of the network could be modulated separately. Moreover background ATP levels and the control of ATP release may vary greatly in different parts of the network. Overall the lack of effect of a blanket block suggests that, under basal conditions seen in the slice, no specific effect dominates but rather the different purinergic effects act in balance to prevent changes in firing of Purkinje cells. It may be that only under conditions of motor activity or other factors which change the balance of firing would it be possible to detect specific effects of one or other site of purinergic activity.

In conclusion, the present study provides the first demonstration of opposite effects of presynaptic P2X and P2Y receptors on GABA release from interneurones adding to previous observations of the role of nucleotides, acting at different levels, on GABAergic transmission in the cerebellum. These sites of purinergic modulation may be individually controlled physiologically with ATP being delivered locally from different sources under specific conditions of motor control. However, the fact that both the firing of interneurones and release from their terminals, as well as postsynaptic GABA receptor function are controlled by endogenous ATP via P2X and P2Y receptors, strongly suggests that interactions of the purinergic and GABAergic systems may be able to control cerebellar output to a very refined level. With P2Y receptors controlling intracellular

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

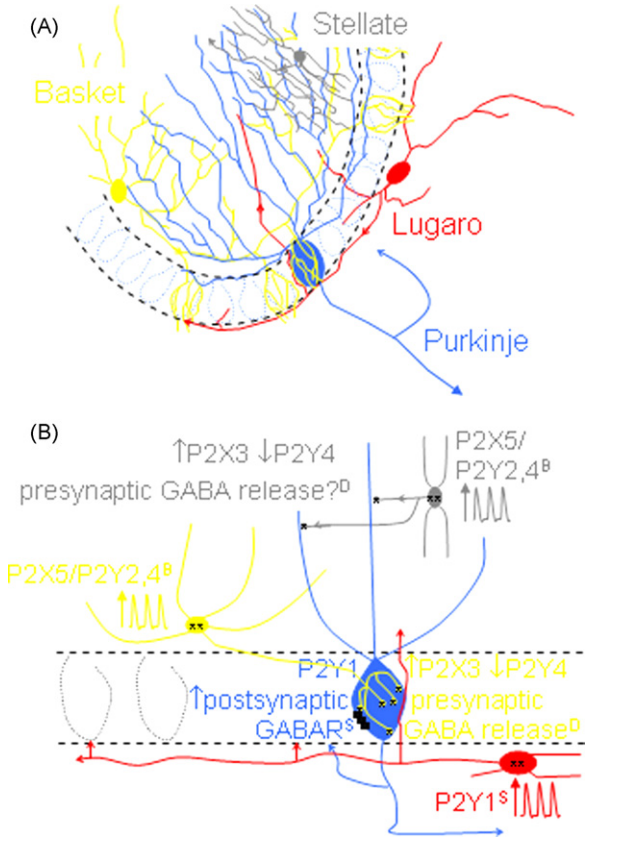


Figure 7 Loci of purinergic modulation of GABAergic transmission in the cerebellar cortex. (A) Adapted from Ramon y Cajal to show the anatomical relationship between the main GABAergic neurons in the cerebellar cortex. (B) Schematic version of above summarizing the loci of P2 receptor modulation with reference to previous publications. Thus, even if restricting analysis to the GABAergic circuitry P2 receptors have been shown to:

(i) Increase the firing of stellate and basket cells (P2X5; P2Y2; P2Y4).
(ii) Increase firing of Lugaro cells (P2Y1).
(iii) Increase conductance of postsynaptic GABA receptors on Purkinje cells (P2Y1).
(iv) Either increase (P2X3) or decrease GABA release from basket cell terminals (P2Y4).

Thus, the end effect on the firing rate of a particular Purkinje cell will depend on the ATP release at particular locations and, if the number of ATP receptors can be modulated, the history of the circuit. This suggests a complex role for purinergic receptors in modulating Purkinje cell output. Numbers in square brackets on the figure refer to references in list.

Ca^{2+} pathways at lower ATP concentrations and P2X receptors dominating at higher concentrations via control of Ca^{2+} influx, the network would be well adapted for fine control of Purkinje cell function under different conditions. Specifically the present study demonstrates, for the first time, the importance of P2X3-containing receptors and P2Y4 receptors on the terminals of basket cells in controlling GABA release.

Acknowledgments

This work was supported by project grants from Wellcome Trust (Ref 057997) and BBSRC to FAE and Fundação para a Ciência e para a Tecnologia (POCI/SAU-NEU/59135/2004). RJR was in receipt of an FCT PhD fellowship. RJR and RG-V were supported at UCL by Marie Curie Training Fellowships.

References

[1] R.A. Cunha, J.A. Ribeiro, Purinergic modulation of [^{3}H]GABA release from rat hippocampal nerve terminals, Neuropharmacology 39 (2000) 1156–1167.
[2] L. Giraldez, M. Diaz-Hernandez, R. Gomez-Villafuertes, J. Pintor, E. Castro, M.T. Miras-Portugal, Adenosine triphosphate and diadenosine pentaphosphate induce [Ca(2+)](i) increase in rat basal ganglia aminergic terminals, J. Neurosci. Res. 64 (2001) 174–182.
[3] M. Díaz-Hernández, R. Gómez-Villafuertes, F. Hernando, J. Pintor, M.T. Miras-Portugal, Presence of different ATP receptors on rat midbrain single synaptic terminals. Involvement of the $P2X_3$ subunits, Neurosci. Lett. 301 (2001) 159–162.
[4] S. Hugel, R. Schlichter, Presynaptic P2X receptors facilitate inhibitory GABAergic transmission between cultured rat spinal cord dorsal horn neurons, J. Neurosci. 20 (2000) 2121–2130.
[5] R. Gomez-Villafuertes, J. Gualix, M.T. Miras-Portugal, Single GABAergic synaptic terminals from rat midbrain exhibit functional P2X and dinucleotide receptors, able to induce GABA secretion, J. Neurochem. 77 (2001) 84–93.
[6] K. Inoue, ATP receptors for the protection of hippocampal functions, Jpn. J. Pharmacol. 78 (1998) 405–410.
[7] B. Sperlágh, A. Köfalvi, J. Deuchars, et al., Involvement of $P2X_7$ receptors in the regulation of neurotransmitter release in the rat hippocampus, J. Neurochem. 81 (2002) 1196–1211.
[8] B.S. Khakh, G. Henderson, ATP receptor mediated enhancement of fast excitatory neurotransmitter release in the brain, Mol. Pharmacol. 54 (1998) 372–378.
[9] J.A. Sim, S. Chaumont, J. Jo, et al., Altered hippocampal synaptic potentiation in P2X4 knock-out mice, J. Neurosci. 26 (August (35)) (2006) 9006–9009, *J Neurosci*. **2006**; 26: 9006–9009.
[10] S. Kirischuk, V. Matiash, A. Kulik, N. Voitenko, P. Kostyuk, A. Verkhratsky, Activation of p2-purino-α1-adreno and H1-histamine receptors triggers cytoplasmic calcium signalling in cerebellar Purkinje neurons, Neuroscience 73 (1996) 643–647.
[11] J.W. Deitmer, J. Brockhaus, D. Casel, Modulation of synaptic activity in Purkinje neurons by ATP, Cerebellum 5 (2006) 49–54.
[12] Y.H. Jo, R. Schlichter, Synaptic corelease of ATP and GABA in cultured spinal neurons, Nat. Neurosci. 2 (1999) 241–245.
[13] R.A. Cunha, J.A. Ribeiro, ATP as a presynaptic modulator, Life Sci. 68 (2000) 119–137.
[14] H.S. Engelman, A.B. MacDermott, Presynaptic ionotropic receptors and control of transmitter release, Nat. Rev. Neurosci. 5 (2004) 135–145.
[15] B.S. Khakh, G. Henderson, Hyperpolarization-activated cationic currents (I_h) in neurones of the trigeminal mesencephalic nucleus of the rat, J. Physiol. [Lond.] 510 (1998) 695–704.
[16] M. Hausser, B.A. Clark, Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration, Neuron 19 (1997) 665–678.
[17] I. Dean, S.J. Robertson, F.A. Edwards, Serotonin drives a novel GABAergic synaptic current recorded in rat cerebellar Purkinje cells: a Lugaro cell to Purkinje cell synapse, J. Neurosci. 23 (2003) 4457–4469.

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006

[18] V.J. Balcar, Y. Li, S. Killinger, M.R. Bennett, Autoradiography of P2X ATP receptors in the rat brain, Br. J. Pharmacol. 115 (1995) 302—306.
[19] R. Kanjhan, G.D. Housley, L.D. Burton, et al., Distribution of the $P2X_2$ receptor subunit of the ATP-gated ion channels in the rat central nervous system [In Process Citation], J. Comp. Neurol. 407 (1999) 11—32.
[20] K.T. Lê, P. Villeneuve, A.R. Ramjaun, P.S. McPherson, A. Beaudet, P. Séguéla, Sensory presynaptic and widespread somatodendritic immunolocalization of central ionotropic P2X ATP receptors, Neuroscience 83 (1998) 177—190.
[21] P. Séguéla, A. Haghighi, J.J. Soghomonian, E. Cooper, A novel neuronal P2X ATP receptor ion channel with widespread distribution in the brain, J. Neurosci. 16 (1996) 448—455.
[22] F. Saitow, T. Murakoshi, H. Suzuki, S. Konishi, Metabotropic P2Y purinoceptor-mediated presynaptic and postsynaptic enhancement of cerebellar GABAergic transmission, J. Neurosci. 25 (2005) 2108—2116.
[23] J. Brockhaus, D. Dressel, S. Herold, J.W. Deitmer, Purinergic modulation of synaptic input to Purkinje neurons in rat cerebellar brain slices, Eur. J. Neurosci. 19 (2004) 2221—2230.
[24] P. Monsivais, B.A. Clark, A. Roth, M. Hausser, Determinants of action potential propagation in cerebellar Purkinje cell axons, J. Neurosci. 25 (2005) 464—472.
[25] Z. Xiang, G. Burnstock, Changes in expression of P2X purinoceptors in rat cerebellum during postnatal development, Brain Res. Dev. Brain Res. 156 (2005) 147—157.
[26] F.A. Edwards, A. Konnerth, B. Sakmann, T. Takahashi, A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system, Pflugers Arch. 414 (1989) 600—612.
[27] G.R. Phillips, J.K. Huang, Y. Wang, et al., The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution, Neuron 32 (2001) 63—77.
[28] R.J. Rodrigues, T. Almeida, P.J. Richardson, C.R. Oliveira, R.A. Cunha, Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus, J. Neurosci. 25 (2005) 6286—6295.
[29] M. Díaz-Hérnández, M.F. Pereira, J. Pintor, R.A. Cunha, J.A. Ribeiro, M.T. Miras-Portugal, Modulation of the rat hippocampal dinucleotide receptor by adenosine receptor activation, J. Pharmacol. Exp. Ther. 301 (2002) 441—450.
[30] C. Virginio, G. Robertson, A. Surprenant, R.A. North, Trinitrophenyl-substituted nucleotides are potent antagonists selective for $P2X_1$, $P2X_3$, and heteromeric $P2X_{2/3}$ receptors, Mol. Pharmacol. 53 (1998) 969—973.
[31] J.L. Boyer, T. Romero-Avila, J.B. Schachter, T.K. Harden, Identifiaction of competitive antagonists of the $P2Y_1$ receptor, Mol. Pharmacol. 50 (1996) 1323—1329.
[32] G. Price, S.J. Robertson, F.A. Edwards, Long-term potentiation of glutamatergic synaptic transmission induced by activation of presynaptic P2Y receptors in the rat medial habenula nucleus, Eur. J. Neurosci. 17 (2003) 844.
[33] N.L. Stricker, R.L. Huganir, The PDZ domains of mLin-10 regulate its trans-Golgi network targeting and the surface expression of AMPA receptors, Neuropharmacology 45 (2003) 837—848.
[34] Y.H. Jo, L.W. Role, Purinergic and GABAergic co-transmission in lateral hypothalamus, Soc. Neurosci. Abs. (2000), 716.19.
[35] T.D. White, W.F. MacDonald, Neural release of ATP and adenosine, Ann. N Y Acad. Sci. 603 (1990) 287—298.
[36] P. Bezzi, A. Volterra, A neuron-glia signalling network in the active brain, Curr. Opin. Neurobiol. 11 (2001) 387—394.
[37] P.G. Haydon, GLIA: listening and talking to the synapse, Nat. Rev. Neurosci. 2 (2001) 185—193.
[38] E.S. Vizi, J.P. Kiss, B. Lendvai, Nonsynaptic communication in the central nervous system, Neurochem. Int. 45 (2004) 443—451.
[39] S. Koizumi, K. Fujishita, M. Tsuda, Y. Shigemoto-Mogami, K. Inoue, Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 11023—11028.
[40] E.A. Newman, Glial cell inhibition of neurons by release of ATP, J. Neurosci. 23 (2003) 1659—1666.
[41] J.M. Zhang, H.K. Wang, C.Q. Ye, et al., ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression, Neuron 40 (2003) 971—982.
[42] C. Hervas, R. Perez-Sen, M.T. Miras-Portugal, Presence of diverse functional P2X receptors in rat cerebellar synaptic terminals, Biochem. Pharmacol. 70 (2005) 770—785.
[43] Y. Geinisman, H.J.G. Gundersen, E. Van der Zee, M.J. West, Unbiased stereological estimation of the total number of synapses in a brain region, J. Neurocytol. 25 (1996) 805—819.
[44] D.A. Rusakov, E. Harrison, M.G. Stewart, Synapses in hippocampus occupy only 1—2% of cell membranes and are spaced less than half-micron apart: a quantitative ultrastructural analysis with discussion of physiological implications, Neuropharmacology 37 (1998) 513—521.
[45] G.P. Connolly, Differentiation by pyridoxal 5-phosphate, PPADS and IsoPPADS between responses mediated by UTP and those evoked by alpha, beta-methylene-ATP on rat sympathetic ganglia, Br. J. Pharmacol. 114 (1995) 727—731.
[46] D. Moore, J. Chambers, H. Waldvogel, R. Faull, P. Emson, Regional and cellular distribution of the P2Y(1) purinergic receptor in the human brain: striking neuronal localisation, J. Comp. Neurol. 421 (2000) 374—384.
[47] D. Houston, M. Ohno, R.A. Nicholas, K.A. Jacobson, T.K. Harden, $^{[32P]}$2-iodo-N6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate ([32P]MRS2500), a novel radioligand for quantification of native P2Y1 receptors, Br. J. Pharmacol. 147 (2006) 459—467.
[48] J. Mateo, M. García-Lecea, M.T. Miras-Portugal, E. Castro, Ca^{2+} signals mediated by P2X-type purinoceptors in cultured cerebellar Purkinje cells, J. Neurosci. 18 (1998) 1704—1712.

Please cite this article in press as: R. Donato, et al., GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx, Cell Calcium (2008), doi:10.1016/j.ceca.2008.03.006