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The novel NTPDase inhibitor sodium polyoxotungstate (POM-1) inhibits ATP breakdown but also blocks central synaptic transmission, an action independent of NTPDase inhibition

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ABSTRACT

Understanding the mechanisms and properties of purinergic signalling would be greatly assisted by the discovery of subtype selective and potent inhibitors of the NTPDase enzymes, which metabolise nucleotides such as ATP and ADP in the extracellular space. Currently ARL 67156 is the best available NTPDase inhibitor, but its relatively poor efficacy means that negative results are difficult to interpret. POM-1 (sodium polyoxotungstate) is a novel NTPDase inhibitor, which has shown promising results with the inhibition of recombinant NTPDases 1, 2 and 3. We have tested the effectiveness and physiological effects of POM-1 with cerebellar and hippocampal slices. Using the malachite green phosphate assay, HPLC and biosensor measurements we have found that POM-1 is more effective at blocking ATP breakdown in cerebellar slices than ARL 67156. The site of inhibition is at the first step of the breakdown cascade (conversion of ATP to ADP) and the effects of POM-1 appear readily reversible. However, POM-1 has multiple effects on synaptic transmission. At the cerebellar parallel fibre-Purkinje cell (PF) synapse POM-1 produced a long lasting inhibition of transmission, which was preceded in a minority of synapses by a transient increase in PF excitatory postsynaptic potential (EPSP) amplitude (~20%). This increase in PF EPSP amplitude appears to result from a reduction in the tonic activation of presynaptic A_1 receptors, consistent with POM-1 preventing the breakdown of ATP to adenosine. The reduction in PF EPSP amplitude does not however appear to result from NTPDase inhibition as it persists when both adenosine and ATP (P2Y and P2X) receptors are blocked. An increase in paired pulse ratio and a reduction in presynaptic volley amplitude suggest that there is a presynaptic component of POM-1 action which reduces glutamate release. POM-1 produced similar inhibition at climbing fibre synapses and at hippocampal CA1 pyramidal synapses. Thus although POM-1 is more effective than ARL 67156 at blocking ATP breakdown its usefulness is limited by off-target actions on synaptic transmission.

1. Introduction

Purinergic signalling is involved in a number of important physiological and pathological processes including respiration, neuroprotection, locomotion and development (for review see Ackland et al., 2007; Rudolphi et al., 1992; Schwarzschild et al., 2006; Zimmermann, 2006; Burnstock, 2007a). It is a complex signalling system, as extracellular nucleotides such as ATP and ADP can activate a multitude of P2 receptors (P2Y and P2X; Khakh, 2001; Burnstock, 2007b) and their metabolite adenosine can activate a variety of P1 receptors (A_1, $A_{2A,B}$ and A_3; Fredholm et al., 2000). Extracellular adenosine can also arise in the extracellular space from sources other than extracellular ATP such as via gap junctions, cytoplasmic translocation via transporters and possibly by exocytosis (Latini and Pedata, 2001; Wall and Dale, 2007). To fully define the roles that individual species play in purinergic signalling, it would be useful to prevent the extracellular metabolism of purines such as ATP and ADP. This has proven difficult as there are currently no potent inhibitors (for example see Wall and Dale, 2007; Frenguelli et al., 2007).

Nucleotides such as ATP and ADP are metabolised in the extracellular space by a family of ecto-nucelotidases (NTPDases) which have different substrate specificities, distributions and therefore roles in purinergic signalling (for review see Zimmermann, 2000). This family of enzymes includes NTPDase 1 which hydrolyses ATP and ADP equally well, NTPDase 2 which has a much higher preference for ATP than ADP whereas NTPDase 3 has intermediate properties, preferably hydrolyzing ATP (5:1). Understanding the roles of these various enzymes would be greatly assisted by the presence of potent and subtype-selective NTPDase inhibitors.

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Currently the most effective drug is ARL 67156 which is relatively weak (no action on NTPDase 2; K_i of 27 and 112 μM on NTPDases 1 and 3, Müller et al., 2006).

Recently Müller et al. (2006) have described the actions of a new class of NTPDase inhibitors, the polyoxometalates. polyoxotungstate are anionic complexes that contain transition metal ions such as tungsten bridged by oxygen atoms. In particular polyoxotungstate 1 ($Na_6[H_2W_{12}O_{40}]$; POM-1) is of interest as it is commercially available, inexpensive and a more potent inhibitor of recombinant NTPDases than ARL 67156. POM-1 has a reported K_i of 2.58 μM at NTPDase 1, 28.8 μM at NTPDase 2 and 3.26 μM at NTPDase 3 (Müller et al., 2006). POM-1 has been used in physiological studies, where it was found to increase the size of cardiac infarct size following ischemia and abolish the protective effects of cardiac and renal ischemic preconditioning (Köhler et al., 2007; Grenz et al., 2007). These effects appear to result from reduced ATP breakdown to cyto-protective adenosine.

We were interested in the effects of POM-1 at synapses within the central nervous system. In particular, determining whether POM-1 effectively reduces extracellular ATP metabolism without producing any off-target actions on neurotransmission. In many parts of the brain, tonic activation of presynaptic A_1 receptors by endogenous extracellular adenosine inhibits synaptic transmission. The source of this adenosine could be extracellular ATP but this often remains unclear as the blockers of NTPDases are weak (for example see Wall and Dale, 2007). We have applied POM-1 to cerebellar slices and found that it is an effective blocker of ATP breakdown but its usefulness in intact preparations is limited by inhibition of glutamatergic synaptic transmission, an action independent of NTPDases, in both cerebellum and hippocampus.

2. Methods and materials

2.1. Cerebellar slice preparation

Slices of cerebellum (parasagittal and transverse, 400 μm) were prepared from male 21–28 day-old Wistar rats, as previously described (Wall and Dale, 2007). In accordance with Schedule 1 of the U.K. Animals (Scientific Procedures) Act (1986), rats were killed by cervical dislocation and then decapitated. Slices were cut on a Microm HM 650V microslicer in cold (2–4 °C) high Mg^{2+}, low Ca^{2+} aCSF, composed of (mM): 127 NaCl, 1.9 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 10 D-glucose (pH 7.4 when bubbled with 95% O_2 and 5% CO_2). Slices were stored in normal aCSF (1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$) at room temperature for 1–6 h before recording.

2.2. Hippocampal slice preparation

Saggittal hippocampal slices (400 μM) were obtained from 16 to 22 day-old Sprague–Dawley rats of either sex using similar methods to above (slices were cut in ice-cold 11 mM Mg^{2+}, 2 mM Ca^{2+} aCSF; Dale et al., 2000; Pearson et al., 2001; Etherington and Frenguelli, 2004). The composition of the normal aCSF solution for recording and storage of hippocampal slices was (in mM): 124 NaCl, 2 $CaCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 D-glucose, 1 $MgSO_4$, pH 7.4 with 95% O_2 and 5% CO_2.

2.3. Recording from cerebellar and hippocampal slices

An individual slice was transferred to a recording chamber, submerged in aCSF and perfused at 5–6 ml/min (30–33 °C). The slice was placed upon a suspended grid to allow perfusion of the slice from above and below and thus reduce the likelihood of hypoxia. All solutions were vigorously bubbled (95% O_2 and 5% CO_2) and all tubing had low gas permeability (Tygon). For the stimulation of parallel fibre-Purkinje cell (PF) field (f) EPSPs, or climbing fibre-Purkinje cell (CF) fEPSPs, square voltage pulses (2–8 V, 200 μs duration) were delivered by an isolated pulse stimulator (Model 2100 AM systems) via a concentric bipolar metal stimulating electrode (FHC) placed on the surface of the slice. For the extracellular recording of PF fEPSPs, an electrode (aCSF filled microelectrode) was placed on the same track along which the parallel fibres travel in transverse slices (for example see Yuan and Atchison, 1999; Wall et al., 2007). Parallel fibre fEPSP amplitude was estimated by subtracting what remained in 10 μM CNQX or 5 mM kynurenate from control potentials. Confirmation of PF EPSP identity was achieved by evoking pairs of fEPSPs (interval 50 ms) and observing facilitation (20–30%) and by examining the pharmacological profile (inhibition by A_1, $GABA_B$ and mGluR4 receptor agonists). Climbing fibre fEPSPs were recorded from parasagittal slices by stimulating the white matter and recording in the molecular layer. The fEPSPs were identified by the presence of paired pulse depression and were blocked by either 10 μM CNQX or 5 mM kynurenate. The large depolarisation produced by climbing fibre EPSPs leads to contamination of the fEPSPs with population spikes (Hashimoto and Kano, 1998) and thus the paired pulse ratio cannot be used to elucidate a pre or postsynaptic action. Cerebellar fEPSPs were recorded with a CED Micro 1401 controlled by Spike 2 software (Vs 6.01 Cambridge Electronic Design). For hippocampal slices, fEPSPs were recorded from stratum radiatum of area CA1, via aCSF-filled recording electrodes, in response to stimulation (100 μs pulses at 15 s intervals; bipolar Teflon-coated tungsten wire; ~100 μm in diameter) of the Schaffer collateral–commissural fibre pathway. The recording and analysis of hippocampal EPSPs were under the control of LTP software (courtesy of Dr. Bill Anderson & Professor Graham Collingridge, University of Bristol, http://www.ltp-program.com/indexLtp24.htm; Anderson and Collingridge, 2001).

2.4. Biosensor characteristics

Biosensors were obtained from Sarissa Biomedical Ltd. (Coventry, UK). In brief adenosine (ADO) biosensors consisted of three entrapped enzymes (adenosine deaminase, nucleoside phosphorylase and xanthine oxidase) within a matrix that was deposited around a Pt or Pt/Ir (90/10) wire etched to 25–50 μM (Llaudet et al., 2003). The biosensor had an exposed length of 500 μm that was coated with enzymes and thus capable of detecting purines. Biosensors had an additional screening layer that greatly reduced the responses to non-specific electro-active interferents (such as 5-HT, dopamine, noradrenaline and ascorbate). Screened null sensors, possessing the matrix but no enzymes, were used to control for the release of any non-specific electroactive interferents. The ATP biosensor consisted of the entrapped enzymes glycerol kinase and glycerol-3-phosphate oxidase (Llaudet et al., 2005). Glycerol (2 mM) was included in solutions as glycerol is a co-substrate required for ATP detection.

Biosensors were calibrated with known concentrations of adenosine and ATP (10 μM typically giving responses of 2–3 nA for adenosine and 1.5 nA for ATP). Calibration was performed before the slice was present in the perfusion chamber and after the experiment (following slice removal), this allowed quantification of any rundown in sensitivity during the experiment. Previous reports have detailed the properties of ADO and ATP biosensors (Llaudet et al., 2003, 2005): selectivity, linear responses to increasing analyte concentration and rapid response. We have used adenosine biosensors to give a measure of the overall purine tone (as they will detect adenosine, inosine and hypoxanthine) and have not quantified the contribution of individual purines (see Wall et al., 2007; Frenguelli et al., 2007). All measurements were made relative to a null sensor. POM-1 did not reduce the sensitivity of either adenosine or ATP biosensors ($n = 3$).

2.5. Determination of ATP breakdown inhibition using the malachite green assay

To assess the efficacy of POM-1 as a blocker of extracellular ATP breakdown, we used the convenient phosphate assay of Carter and Karl (1982) (see Frenguelli et al., 2007). Cerebellar slices (400 μm) were prepared from 21–28 day male Wistar rats. Slices were kept in oxygenated aCSF solution at room temperature for 1 h prior to being placed into individual wells in a 24 well plate. The cerebellar slices were then washed five times with phosphate-free aCSF (comprising (in mmol/L): 3.1 KCl, 126 NaCl, 26 $NaHCO_3$, 10 D-glucose, 1 $CaCl_2$, 2 $MgCl_2$). The slices (one per well) were then immersed in 400 μl of test solution (phosphate-free aCSF with either 25 or 100 μmol/L ATP). To prevent hypoxia, 5% CO_2 and 95% O_2 were blown across the surface of each well. The multi-well plate was placed on a shaker so that there was gentle agitation of the slices and good mixing within the wells. To maintain the health of the slices each experimental run was restricted to 30 min, with 25 μl aliquots being removed at 0, 1, 3, 5, 10, 15, 20, 25 and 30 min for phosphate analysis. The 25 μl samples from control and ARL 67156 studies were diluted 4 fold before the assay was performed. However, unlike ARL 67156, POM-1 reduced the linear range of the standard phosphate curve thus greater dilutions were made at longer time points to ensure that the concentration of phosphate was within the linear range. The rate of phosphate production (measured from the slope of the graph of [phosphate] versus time) was compared in the control to the rate in the presence of POM-1 or ARL 67156. A timed control was also performed whereby the rate of phosphate production was compared for two successive runs without the addition of POM-1 or ARL 67156. This resulted in a small reduction of the rate of phosphate production between runs of $4.1 \pm 4.5\%$ ($n = 8$). This small decrease in the control was subtracted from the decreases caused by POM-1 and ARL 67156 to give the net reduction in phosphate production due to the inhibition of the ectoATPases.

2.6. Determination of ATP breakdown inhibition using HPLC

To investigate the site of action of polyoxotungstate in blocking ATP breakdown, the breakdown of etheno-ATP (ε-ATP) was measured using HPLC (Wall and Dale, 2007). Individual 400 μm cerebellar slices were incubated (on a shaker, at room temperature) in 400 μl of phosphate-free aCSF containing 50 μM ε-ATP (20 nmol of ε-ATP) with or without inhibitors (POM-1). At 0, 10, 25 and 45 min, 50 μl samples were taken and snap frozen with dry ice.

Samples were thawed out and diluted 1 in 10. HPLC analysis was performed with a Luna C8 (2) reverse phase column and a Thermo Separation Products HPLC gradient pump (P2000) and fluorescence detector (FL3000). The mobile phase

consisted of 20 mM potassium phosphate pH 6.0 (A) and 75% potassium phosphate pH 6.0 and 25% methanol (B). A concave gradient was run from 100% A to 100% B in 10 min. ε-ATP typically eluted around 3.5 min, ε-ADP around 4 min, ε-AMP 5 min and ε-adenosine around 9 min. The column was re-equilibrated with solution (A) for 10 min between runs. To quantify ε-ATP breakdown, the relative proportions of the breakdown products were obtained from peak areas.

2.7. Drugs

All drugs were made up as 10–100 mM stock solutions, stored frozen and then thawed and diluted with aCSF on the day of use. Sodium polyoxotungstate (POM-1) was dissolved in distilled water. POM-1 was initially supplied by Professor Christa Müller and then further quantities were obtained from Sigma. Adenosine and 8-cyclopentyltheophylline (8 CPT) were obtained from Sigma. ARL 67156 was purchased from Tocris-Cookson and kynurenate was obtained from Ascent Scientific. ATP was purchased from Roche and etheno-ATP (ε-ATP) was purchased as a 5 mM solution from Invitrogen.

2.8. Analysis

Statistical significance was assessed using either the paired or unpaired *t*-test or by a chi squared test. Data is presented as a mean ± SEM.

3. Results

3.1. POM-1 is a more effective inhibitor of extracellular ATP breakdown than ARL 67175

To assess the efficacy of POM-1 as an NTPDase inhibitor, the inhibition of ATP hydrolysis by cerebellar slices was measured using the malachite green phosphate assay and compared to the inhibition produced by the standard NTPDase inhibitor ARL 67156 (Fig. 1). At 100 μM, POM-1 produced significantly ($P < 0.01$) greater inhibition of ATP (100 μM) breakdown than 100 μM ARL 67156 (mean inhibition POM-1, 57.4 ± 3.8%; ARL 67156, 13.9 ± 4.3%, $n = 7$). Decreasing the concentration of ATP (to 25 μM) greatly increased the efficacy of ARL 67156 but had no significant ($P > 0.05$) effect on the efficacy of POM-1 (mean inhibition, POM-1, 68 ± 7%; ARL 67156, 43 ± 7.5%, $n = 5$). At lower concentrations (10 μM) POM-1 had only weak effects on the breakdown of ATP (mean inhibition of 25 μM ATP breakdown 17.8 ± 2.3%; mean inhibition of 100 μM ATP, 11.3 ± 11.4%, $n = 5$). Thus, in agreement with the data from recombinant NTPDases, POM-1 is a more potent inhibitor of ATP metabolism than ARL 67156.

3.2. POM-1 acts to block ATP breakdown

HPLC analysis of the effects of POM-1 on cerebellar ATP breakdown was performed to assess the position in the breakdown cascade that POM-1 acts. Like ARL 67156 (Wall and Dale, 2007), POM-1 slowed the breakdown of ATP and significantly slowed the accumulation of ATP metabolites: ADP, AMP and adenosine (Fig. 1E; 6 control slices and 5 slices in the presence of 100 μM POM-1). Thus it appears that POM-1 acts at the first stage of ATP breakdown, the conversion of ATP to ADP.

3.3. The actions of POM-1 on ATP breakdown are readily reversible

To investigate whether the inhibition of cerebellar ATP breakdown was reversible, adenosine (ADO) biosensors and null sensors were placed onto the surface of the molecular layer of cerebellar slices to measure the real-time production of the ATP metabolites adenosine and inosine following the extracellular breakdown of applied ATP. The inflow was changed from control saline to 100 μM ATP for 3 min (see Wall et al., 2007). Following the application of 100 μM ATP, a current (300–500 pA) was detected on the ADO sensor (Fig. 2A). Although the ADO sensor will detect both adenosine and inosine, in previous studies we have found that only ~7% of the current arises from inosine production following the application

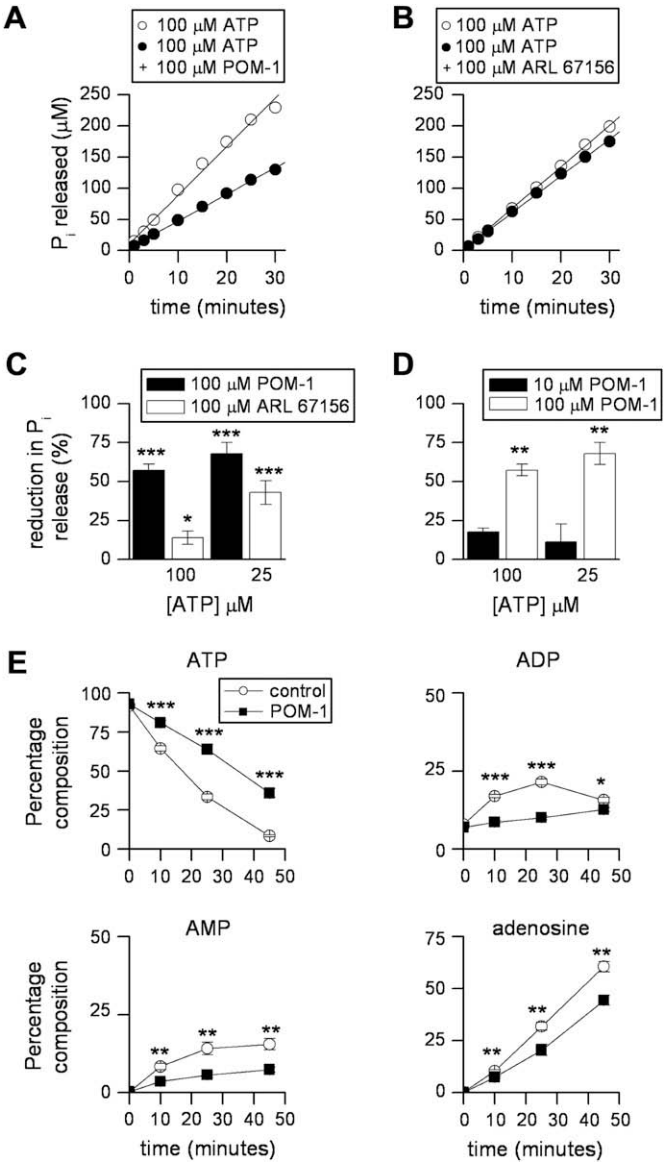

Fig. 1. POM-1 is a more effective inhibitor of cerebellar ATP breakdown than ARL 67156. (A, B) The time-dependent breakdown of ATP by cerebellar slices measured through the accumulation of inorganic phosphate (P_i) using the malachite green assay. Application of POM-1 (100 μM) substantially lowered the rate of 100 μM ATP breakdown (A) whereas ARL 67156 (100 μM) was much less effective (B). (C) Graph summarizing the inhibitory actions of 100 μM POM-1 and 100 μM ARL 67156 on the breakdown of 100 μM ATP (7 slices) measured with the malachite green assay. (D) Graph summarizing the inhibitory actions of 10 and 100 μM POM-1 on ATP breakdown (25 and 100 μM ATP, 5–7 slices). (E) Graphs summarizing HPLC data illustrating the metabolism of 100 μM e-ATP and the accumulation of metabolites (ADP, AMP and adenosine data from 6 control slices and 5 slices with POM-1 present). Open circles are control and closed squares are with 100 μM POM-1. POM-1 reduces the breakdown of ATP with subsequent reduction in the accumulation of ADP, AMP and adenosine. Significant differences are denoted by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

of ATP to cerebellar slices, with 93% of the signal from adenosine detection (Wall et al., 2007). Assuming all the signal is due to adenosine, then 4.8 ± 0.7 μM of adenosine was produced by application of ATP ($n = 5$ slices). Thus about 5% of the applied ATP was converted to adenosine which is a similar proportion to that observed in previous studies (Wall et al., 2007) and in hippocampal slices (Frenguelli et al., 2007). In the presence of 100 μM POM-1 the production of adenosine was significantly ($P < 0.01$) reduced by ~52% to 2.3 ± 0.2 μM (Fig. 2A and B). This is a similar level of inhibition to that observed with the malachite green phosphate assay (57%). Following the wash of POM-1, the production of adenosine

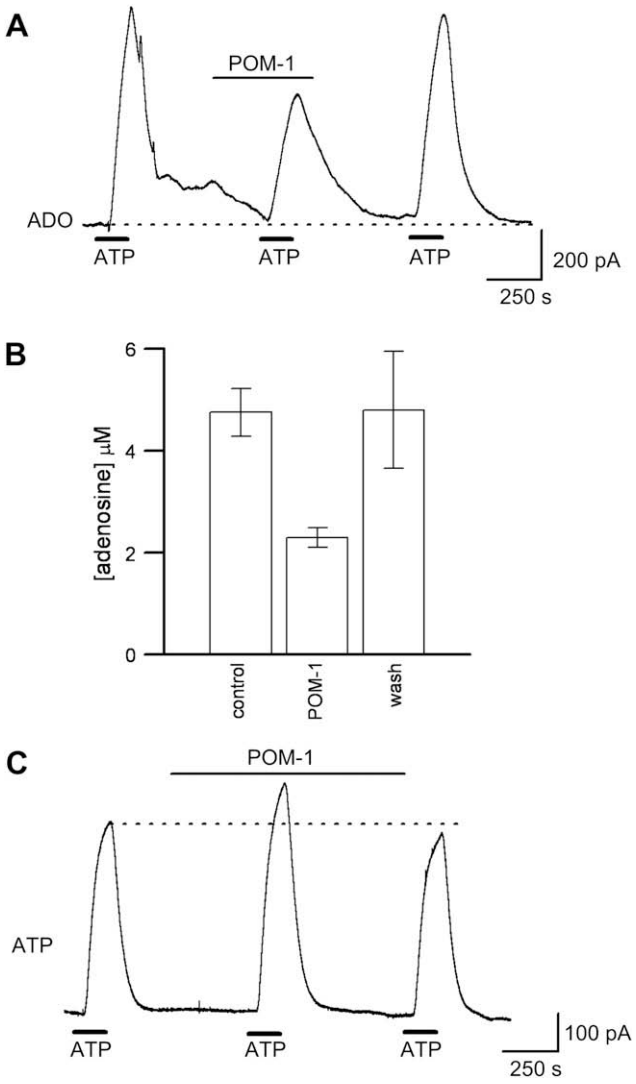

Fig. 2. The POM-1-mediated inhibition of cerebellar ATP breakdown is readily reversible. (A) Trace from an adenosine biosensor (ADO) placed parallel to the surface of the molecular layer. Following application of ATP (100 μM), currents were produced on the adenosine sensor due to the breakdown of ATP to adenosine (and inosine). Application of 100 μM POM-1 reduced the production of adenosine by ~50%, which was reversible upon wash. (B) Graph summarizing data from 5 slices, POM-1 (100 μM) reversibly reduces the production of adenosine by ~50%. (C) Trace from an ATP biosensor (ATP) inserted into the molecular layer. Following application of ATP (100 μM), currents were produced on the ATP sensor. Application of 100 μM POM-1 increased the biosensor current suggesting ATP breakdown occurs before detection. Again the actions of POM-1 were readily reversible upon wash.

recovered to similar levels (4.8 ± 1.15 μM) to that observed in control. Thus the POM-1-mediated inhibition of cerebellar ATP breakdown is readily reversible. In 3 slices, ATP biosensors were inserted into the molecular layer during the application of 100 μM ATP. As previously observed in hippocampal slices (Frenguelli et al., 2007) only a small percentage of the applied ATP was detected (~7%). Addition of 100 μM POM-1 reversibly and significantly ($P < 0.05$) increased the amount of ATP detected by the biosensors by ~20% (from $7.2 \pm 3\%$ to $8.7 \pm 2\%$, Fig. 2C) and thus a proportion of the ATP was broken down in the slice before sensor detection.

3.4. Heterogeneous actions of POM-1 on synaptic transmission at the parallel fibre-Purkinje cell synapse

Although POM-1 appears a potent blocker of NTPDases in the cerebellum, its usefulness will depend on whether it has any off-target actions on neurotransmission. Thus we have investigated the effects of POM-1 on parallel fibre-Purkinje cell (PF) synaptic transmission using field recording. Transmission at parallel fibre to Purkinje cell synapses is inhibited by the activation of presynaptic A_1 receptors (Kocsis et al., 1984; Takahashi et al., 1995; Dittman and Regehr, 1996). Since A_1 receptor antagonists enhance the amplitude of PF excitatory postsynaptic potentials (PF EPSPS) in the majority of cerebellar slices (around 90%), A_1 receptors are continually activated by a tone of extracellular adenosine (Wall et al., 2007; Takahashi et al., 1995; Dittman and Regehr, 1996). If the adenosine tone arises from NTPDase-mediated extracellular ATP breakdown, then POM-1 would be expected to enhance PF EPSP amplitude. At low concentrations (20 μM, $n = 6$ and 30 μM, $n = 3$), POM-1 had no effect on PF fEPSP amplitude (data not shown), although blocking A_1 receptors (8 CPT 1 μM in the presence of POM-1) still increased fEPSP amplitude indicating the continued presence of an adenosine tone. Application of higher concentrations of POM-1 (100 μM), which would be more effective at inhibiting NTPDase, had multiple effects on synaptic transmission (Fig. 3). In 6 of 22 slices POM-1 produced a transient increase in PF fEPSP amplitude which was followed by a long lasting reduction in PF fEPSP amplitude (Fig. 3A). In a further 14 slices, POM-1 produced a delayed but marked reduction in fEPSP amplitude with no transient fEPSP amplitude enhancement (Fig. 3D). In the remaining 2 slices, application of POM-1 for 10–15 min had no effect on fEPSP amplitude (not shown).

3.5. The increase in EPSP amplitude results from a reduction in adenosine tone

Application of POM-1 (100 μM) produced a transient but significant ($P < 0.01$) increase in EPSP amplitude of $22.2 \pm 4.9\%$ in 6 out of 22 slices (Fig. 3A). This increase could be the result of a reduction in the adenosine tone (as less extracellular ATP is converted to adenosine). This is supported by a small but significant ($P < 0.01$) reduction in the paired pulse ratio (from 1.34 ± 0.06 to 1.27 ± 0.11, interval between stimuli 50 ms, $n = 6$; data not shown) during the period when the fEPSP amplitude was increased, suggesting the increase in fEPSP amplitude had a presynaptic component. If the increase in fEPSP amplitude was due to a reduction in adenosine tone, then blocking the A_1 receptors (with 8 CPT) should prevent any increase in fEPSP amplitude. In the presence of 8 CPT (1 μM), 100 μM POM-1 produced no increase in fEPSP amplitude ($n = 15$ slices; Fig. 3B) but either reduced fEPSP amplitude (12 slices) or had no effect on fEPSP amplitude (3 slices). There is a greater than 95% probability that this result did not arise from chance (Chi squared test, one degree of freedom) and thus the enhancement of fEPSP amplitude is prevented by blocking A_1 receptors.

Adenosine biosensors were used to measure any reduction in purine tone produced by POM-1. An adenosine biosensor and null sensor were inserted into the molecular layer. Application of 100 μM POM-1 caused a small but significant ($P < 0.05$) reduction in the extracellular purine tone in 7 out of 21 slices (reduction 63.5 ± 25 pA; Fig. 3C) with no effect on the null sensors. In the other 14 slices there was no change in the purine tone. Taken together, the electrophysiology and biosensor experiments, suggest that the increase in EPSP amplitude observed in some slices results from a reduction in the adenosine tone.

In the majority of slices (14 out of 22) POM-1 did not increase the fEPSP amplitude and thus either did not reduce the adenosine tone, or the increase in fEPSP amplitude was occluded by a simultaneous decrease in fEPSP amplitude. To test this we measured the effects of blocking A_1 receptors following POM-1 application, on those synapses where no initial increase in EPSP amplitude was observed (Fig. 3D). After allowing the effects of POM-1 to reach steady state, 8 CPT (1 μM) was applied and produced an increase in fEPSP amplitude of $38.5 \pm 4\%$ and reduced the paired pulse ratio

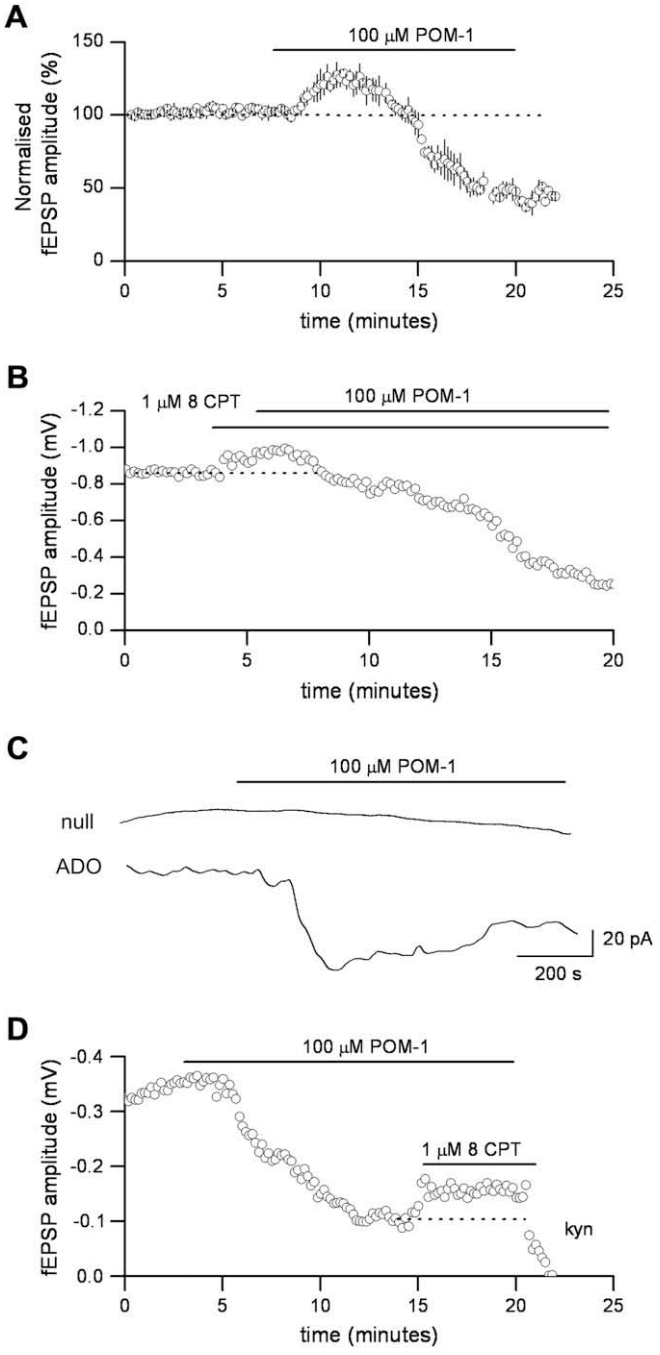

Fig. 3. The actions of POM-1 on synaptic transmission at parallel fibre-Purkinje cell synapses. (A) Graph plotting the pooled and normalized data from 6 slices where application of POM-1 (100 μM) caused a transient increase in EPSP amplitude (~20%) followed by a reduction in EPSP amplitude (~60%). (B) Graph plotting the amplitude of individual fEPSPs against time. Application of 8 CPT (1 μM) increased the amplitude of fEPSPs and prevented any increase in EPSP amplitude mediated by POM-1. However POM-1 still produced a delayed reduction in EPSP amplitude. (C) Traces from an ADO biosensor and null sensor inserted into in the molecular layer of a cerebellar slice. Application of POM-1 reduced the current on the ADO biosensor but had no effect on the null sensor suggesting POM-1 reduced the purine tone. (D) Graph plotting the amplitude of individual fEPSPs against time. Application of POM-1 (100 μM) caused a marked reduction in EPSP amplitude. Addition of 8 CPT (1 μM) increased fEPSP amplitude by ~30% suggesting that the adenosine tone had not been abolished by POM-1. Addition of kynurenic acid (5 mM) blocked synaptic transmission confirming the glutamatergic nature of PF fEPSPs.

from 1.7 ± 0.1 to 1.36 ± 0.2 ($n = 4$). This is comparable to the increase in fEPSP amplitude produced by 8 CPT in control conditions (~46%, Wall et al., 2007). This observation suggests that POM-1 does not reduce the adenosine tone at all synapses and is supported by the lack of effect of POM-1 on the purine tone measured by biosensors in the majority of slices (14 out of 21).

3.6. The inhibition of synaptic transmission is presynaptic and does not appear to result from NTPDase inhibition

In the majority of slices, the application of POM-1 (100 μM) led to a marked and long lasting reduction in PF fEPSP amplitude (16 out of 22 slices). The onset of inhibition was often slow (several minutes) with the degree of inhibition highly variable across synapses (a 10 min application of 100 μM POM-1 reduced fEPSP amplitude by $50.8 \pm 6.9\%$, range 25–84%). Longer applications lead to an almost complete block of synaptic transmission (85–95%, $n = 3$, Fig. 4A). Application of higher concentrations of POM-1 (200–300 μM) sped the onset of inhibition ($n = 3$; not shown). In some slices the inhibition was partly reversible with prolonged washing (20–30 min, Fig. 4A and D) whereas in other slices the inhibition appeared irreversible. This is in stark contrast to the rapidly reversible effects of POM-1 observed in the purine biosensor experiments (Fig. 2).

To investigate the site of action of POM-1, pairs of fEPSPs were evoked and the paired pulse ratio measured. During the POM-1-mediated reduction of PF fEPSP amplitude, the paired pulse ratio was significantly ($P < 0.001$) increased (from 1.35 ± 0.04 to 2.09 ± 0.18, $n = 11$, Fig. 4B) suggesting that a component of the inhibition is presynaptic. However, part of the POM-1 inhibition was mediated by a decrease in the number of active parallel fibres as the presynaptic fibre volley was reduced (mean reduction $18.1 \pm 4.5\%$, Fig. 4C). Application of adenosine (100 μM), which depressed the fEPSP to ~40% control also reduced the presynaptic fibre volley but its effects were small and reversible (~6%, Fig. 4C) and similar to those reported in the hippocampus (Dunwiddie and Miller, 1993).

It is possible that the block of extracellular ATP metabolism produces an accumulation of extracellular ATP leading to presynaptic P2 receptor activation and a subsequent reduction in EPSP amplitude (for example see Armstrong et al., 2002; Mendoza-Fernández et al., 2000). To detect any accumulation of ATP, ATP biosensors were inserted into the molecular layer. As previously reported (Wall et al., 2007), ATP biosensors did not detect an extracellular ATP tone within the slice (no difference between ATP sensor and null sensor). Application of 100 μM POM-1 did not produce a current on the ATP biosensor and thus no accumulation of ATP could be detected ($n = 10$ slices). Furthermore, the POM-1 (100 μM)-mediated reduction in PF fEPSP amplitude was still observed in the presence of P2 receptor antagonists: PPADS (10 μM, $n = 5$), Reactive Blue 2 (RB2, 30 μM, $n = 3$; Fig. 4D) and Brilliant Blue G (BBG, 1–2 μM, $n = 3$; Fig. 4E) applied alone or in combination. Activation of possible P2 receptors with ATP (100–300 μM) or the selective P2X7 receptor agonist Bz-ATP (30 μM), in the presence of 8 CPT (2 μM), had no effect on PF fEPSP amplitude ($n = 3$; not shown). Thus it appears unlikely that the reduction in EPSP amplitude stems from NTPDase inhibition (and subsequent accumulation of ATP). The inhibition may thus represent an off-target action of POM-1.

3.7. POM-1 inhibits synaptic transmission at other glutamatergic synapses

To investigate what effect POM-1 has at other glutamatergic synapses, climbing fibre-Purkinje cell (CF, Fig. 5) and CA1 hippocampal synapses (Fig. 6) were examined. Application of 100 μM POM-1 caused a marked depression in the amplitude of CF fEPSPs ($75.5 \pm 15\%$) in 9 out of 10 slices. POM-1 caused a loss of the contaminating population spikes present in fEPSPs as presumably

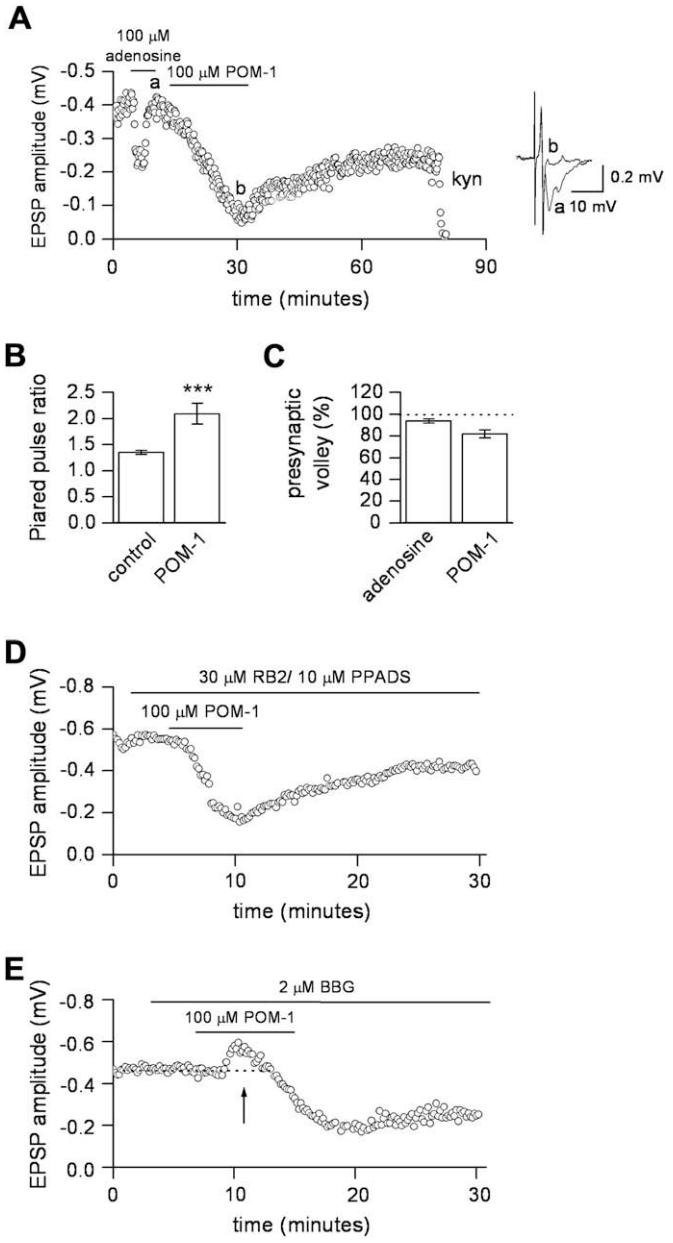

Fig. 4. The reduction in parallel fibre fEPSP amplitude has a presynaptic component and does not appear to result from NTPDase inhibition. (A) Graph plotting the amplitude of individual fEPSPs against time. Application of POM-1 (100 μM) produced a long-lasting inhibition which was partly reversible with prolonged washing. Inset: single fEPSPs from (A) in control (a) and in the presence of 100 μM POM-1 (b). (B) Graph summarizing data from 11 slices. Application of POM-1 (100 μM) produced a significant increase in the paired pulse ratio. (C) Graph summarizing data from 11 slices. Application of POM-1 (100 μM) produced a significant decrease in the presynaptic volley, a larger effect than 100 μM adenosine. (D) Graph plotting the amplitude of individual fEPSPs against time. Application of POM-1 (100 μM) produced a long-lasting inhibition in the present of P2 receptor blockers: PPADs (10 μM) and Reactive Blue 2 (RB2; 30 μM). (E) Graph plotting the amplitude of individual fEPSPs against time. POM-1 produced a transient increase in fEPSP amplitude (arrow) followed by a marked reduction in fEPSP amplitude in the presence of the P2 receptor antagonist Brilliant Blue G (BBG; 2 μM).

EPSP amplitude becomes subthreshold and spikes are no longer fired (Fig. 5B and C).

POM-1 also reduced the fEPSP at hippocampal CA3–CA1 synapses ($n = 11$ slices; Fig. 6A and B) producing an almost complete block of transmission ($88.9 \pm 1.4\%$ inhibition). This inhibition was associated with an $18.0 \pm 3.2\%$ reduction in the presynaptic fibre volley ($n = 7$; Fig. 6C) and a marked increase in paired pulse ratio

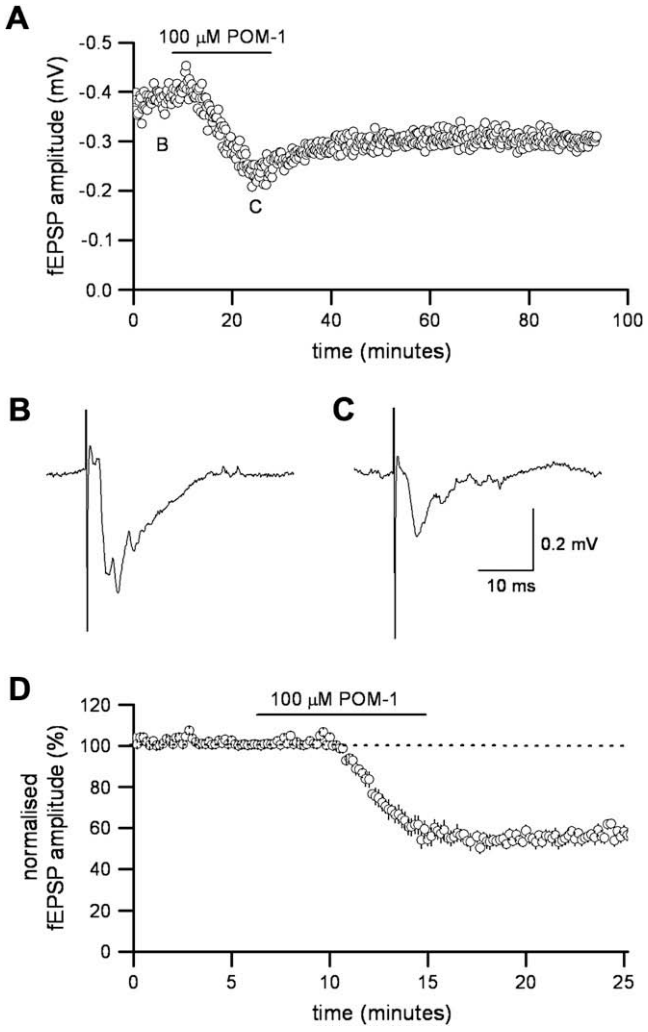

Fig. 5. POM-1 inhibits synaptic transmission at climbing fibre synapses. (A) Graph plotting the amplitude of individual climbing fibre fEPSPs against time. Application of POM-1(100 μM) caused a long lasting reduction in the amplitude of fEPSPs, which did not recover during prolonged wash. (B) A single control fEPSP (from (A)). (C) A single fEPSP in the presence of 100 μM POM-1 (from (A)). (D) Pooled and normalized graph of the effects of POM-1 application on the climbing fibre fEPSP ($n = 5$).

(from 1.71 ± 0.10 to 3.57 ± 0.54; $P = 0.021$, $n = 6$; Fig. 6D). Thus, POM-1 has similar effects at all 3 tested glutamatergic synapses.

4. Discussion

We have investigated the actions of the novel NTPDase inhibitor, polyoxotungstate (POM-1) on the breakdown of extracellular ATP by cerebellar slices. As predicted from studies on recombinant NTPDases (Müller et al., 2006), POM-1 appeared a much more effective blocker than a standard inhibitor (ARL 67156). POM-1 appeared equally effective at reducing the breakdown of both low and high concentrations of ATP (25 and 100 μM, ~60% inhibition), whereas the effectiveness of ARL 67156 was much more dependent on the initial concentration of substrate (ATP) (~14% inhibition with 100 μM ATP; ~44% inhibition with 25 μM ATP). Thus ARL 67156 appears to act as a competitive inhibitor, with higher concentrations of ATP overcoming the block. In contrast, the lack of effect of substrate concentration on the degree of POM-1 inhibition suggests that either POM-1 acts non-competitively or has a much higher affinity for the NTPDases compared to ATP. This remains unclear as studies with recombinant NTPDases only used one concentration of substrate (320 μM ATP, Müller et al., 2006). The

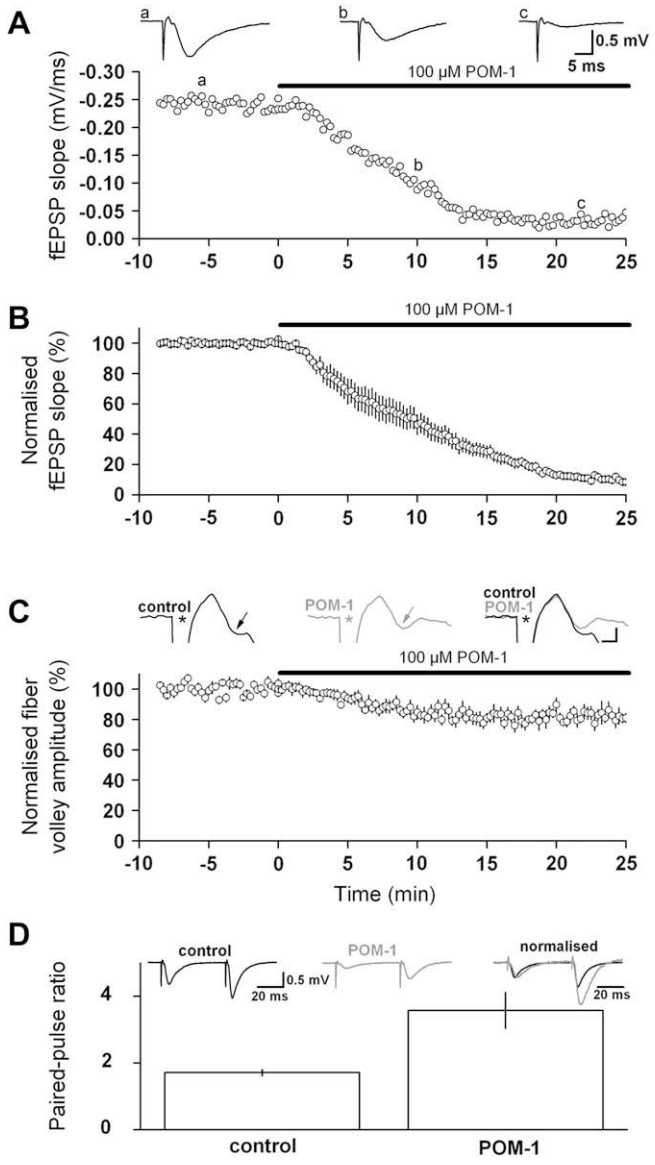

Fig. 6. POM-1 (100 μM) markedly decreased the fEPSP slope at Schaffer collateral-CA1 pyramidal cell synapses. (A) Graph plotting the amplitude of single fEPSPs against time. Application of POM-1 (100 μM) greatly inhibited the fEPSP. Inset: three fEPSPs taken at different times during this experiment ((a) control; (b) 10 min after the start of POM-1 application; (c) 22 min after the start of POM-1 application). (B) Pooled and normalized graph of the effects of POM-1 application on the fEPSP ($n = 11$). (C) Fiber volley amplitude was decreased by ~20% on application of POM-1 ($n = 7$). Insets: fEPSPs from (A) on an expanded scale showing the stimulus artifact (*) and fiber volleys (arrows) in the absence (control) and presence of POM-1 and on superimposition of the two. Note smaller fiber volley in POM-1 (grey). Scale bar measures 0.5 ms and 0.05 mV. (D) POM-1 increased paired pulse facilitation (PPF; 50 ms inter-pulse interval, $n = 6$). Inset: examples of PPF in the absence (control) and presence of POM-1. The superimposed traces on the right show the fEPSPs normalized to the peak of the first fEPSP, allowing more direct comparison of the enhancement of PPF in POM-1 (grey traces).

site of POM-1 inhibition in the enzyme cascade of ATP metabolism appears to be at the first step (ATP to ADP) as there was reduced production of all the downstream metabolites (ADP, AMP and adenosine). A direct comparison of ARL 67156 and POM-1 using HPLC analysis showed similar effects (block of ATP-ADP step, Wall and Dale, 2007) but the effects of ARL 67156 were weaker.

At first sight, there appears to be differences between the HPLC and the biosensor data, with the biosensors suggesting POM-1 blocks ~50% of the adenosine production (following ATP breakdown) whereas the HPLC suggests POM-1 only blocks ~30%. We do not believe that a direct comparison of the two techniques is possible – one is performed in a static bath at defined time points at room temperature, the other in a recording chamber with continuous flow, at 30–32 °C and continuous biosensor measurements. Furthermore, the HPLC measures bulk changes in the medium which are subject to dilution, whilst the biosensors detect changes in a spatially restricted area around the sensor. In addition the HPLC measures the relative proportion of the nucleotides: ATP, ADP, AMP and adenosine. Changes in the total amount of nucleotides are not measured. This is not the case with the biosensor, which detects the absolute amount of adenosine, and the malachite green assay, which detects the absolute amount of phosphate accumulated.

Nonetheless, the conclusion of these various techniques in assessing the actions of POM-1 on cerebellar ATP breakdown suggests that POM-1 could be a useful compound for investigating purine signalling in the central nervous system. However an off-target inhibition of glutamatergic synaptic transmission (discussed later) may greatly reduce its usefulness in intact systems.

4.1. POM-1 reduced the endogenous adenosine tone at a proportion of parallel fibre-Purkinje cell synapses

At some parallel fibre-Purkinje cell (PF) synapses, application of POM-1 caused a transient increase in fEPSP amplitude of ~20%. Several lines of evidence suggest that this increase in amplitude results from a reduction in the endogenous adenosine tone and hence reduced activation of presynaptic A_1 receptors. Firstly, the increase in PF fEPSP amplitude was associated with a reduction in the paired pulse ratio suggesting a presynaptic site of action. Secondly, the enhancement was blocked with the A_1 receptor antagonist 8 CPT. Thirdly, in around one-third of slices examined, POM-1 caused a small reduction in the adenosine biosensor current suggesting a fall in the endogenous purine (adenosine, inosine or hypoxanthine) tone. Previous studies have shown that most of the purine tone in cerebellar slices, measured by biosensors, consists of the metabolites inosine and hypoxanthine rather than adenosine (Wall et al., 2007).

In the majority of cerebellar slices, POM-1 did not produce the expected increase in PF fEPSP amplitude but instead produced a reduction in amplitude. In these synapses blocking A_1 receptors with 8 CPT caused an increase in fEPSP amplitude demonstrating the continued presence of an adenosine tone in the presence of POM-1. Thus POM-1 appears to only reduce the adenosine tone at a subset of synapses. These effects are similar to those observed in previous studies where application of a combination of ARL 67156 and α, β methyleneADP (a nucleotidase inhibitor) had no effect on PF fEPSP amplitude or purine tone at the majority of synapses (Wall et al., 2007). However, as with POM-1, at a minority of synapses the combination of ARL 67156 and α, β methyleneADP increased fEPSP amplitude and reduced the purine tone. Thus, the results with POM-1 and ARL 67156 and α, β methyleneADP are consistent and suggest that at a minority of synapses the adenosine tone arises at least in part from extracellular ATP breakdown. At the other synapses either the NTPDases are not effectively inhibited, other enzymes are responsible for ATP breakdown or the adenosine tone does not arise from extracellular ATP metabolism. It is also possible that some of the heterogeneity stems from differences in slice viability although this seems unlikely as synaptic transmission is intact across all slices.

4.2. POM-1 inhibits synaptic transmission: an action independent of purinergic signalling

At the majority of parallel fibre-Purkinje cell synapses, POM-1 caused a marked and long lasting reduction of fEPSP amplitude. There are several lines of evidence that suggest that this inhibition does not result from NTPDase inhibition. Firstly, a similar degree of

inhibition was observed at all 3 of the tested glutamatergic synapses suggesting a common mechanism. Secondly, the inhibitory actions of POM-1 persisted in the presence of 8 CPT and thus do not result from increased adenosine release possibly due to accumulated ATP overcoming the NTPDase block. Thirdly, it was not possible to prevent the inhibition by blocking P2 receptors thus eliminating the possibility that POM-1 inhibition of NTPDases causes an accumulation of ATP leading to the activation of P2 receptors and the subsequent reduction in EPSP amplitude. For example, activation of P2X7 receptors has been reported to produce a long lasting inhibition of synaptic transmission at the mossy fibre-CA3 pyramidal cell synapse in the hippocampus, an effect reversed with Brilliant Blue G (Armstrong et al., 2002). Activation of P2Y receptors has also been reported to inhibit synaptic transmission in the hippocampus synapses (Mendoza-Fernández et al., 2000). Both P2X7 and P2Y receptors have been reported to be expressed by granule cells (Leon et al., 2006) and thus could conceivably inhibit transmitter release. However ATP biosensors detected no ATP current in the presence of POM-1 and the P2 receptor antagonists PPADS, Reactive Blue 2 and Brilliant Blue G did not reverse the inhibitory actions of POM-1. Furthermore applications of P2 receptor agonists, ATP and BzATP (with A_1 receptors blocked) had no effect on EPSP amplitude at parallel fibre-Purkinje cell synapses. POM-1 also reduced climbing fibre EPSPs and there is no evidence that P2 receptors modulate these synapses. In addition, application of others blockers of ATP, such as ARL 67156 and α, β methyleneADP, did not produce a marked reduction in EPSP amplitude (Wall et al., 2007). Finally, the actions of POM-1 against NTPDases were rapidly reversible, unlike the effects on synaptic transmission. Thus it appears likely that POM-1 inhibition of synaptic transmission is due to off-target effects.

The inhibitory actions of POM-1 appear, at least in part, presynaptic at parallel fibre-Purkinje cell and hippocampal CA1 synapses, as the paired pulse ratio was markedly increased, and the small reduction in presynaptic volley suggests that the number of active presynaptic fibres is reduced. It is unclear whether the fall in the presynaptic volley ($\sim$20%) is sufficient to account for the much greater reduction in EPSP amplitude. We cannot exclude the possibility that POM-1 is inhibiting presynaptic voltage-gated calcium channels, inhibition of which would also depress transmission and increase the paired pulse ratio. The slow onset of inhibition and very slow reversal suggest that POM-1 may have to be internalized into the nerve terminals before it has its off target actions and then it is difficult to washout.

In summary, POM-1 is an effective inhibitor of NTPDases, potentially with an attractive non-competitive mechanism. As such it may serve a useful purpose in recombinant, reduced systems and as a lead compound for other inhibitors. However, its additional, and presently unspecified, actions augur caution in its use in more intact preparations.

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