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Presynaptic internal Ca^{2+} stores contribute to inhibitory neurotransmitter release onto mouse cerebellar Purkinje cells

[1,2]Scott Bardo, [1]Brian Robertson & *,[1]Gary J. Stephens

[1]Neuronal Excitability Group, Biophysics Section, Department of Biological Sciences, Imperial College of Science, Technology & Medicine, London SW7 2BZ

1 Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in mouse Purkinje cells in the presence of 1 μM tetrodotoxin (TTX). Under these conditions, which eliminated Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs), the contribution of Ca^{2+} stores to spontaneous GABA release was examined.

2 The plant alkaloid ryanodine acts as an inhibitor of endoplasmic reticulum ryanodine-sensitive Ca^{2+} release channels (ryanodine receptors) at low micromolar concentrations. Ryanodine effects were confined to a subpopulation of cells tested. At 10 μM ryanodine, 4/12 cells showed a significant increase in mean mIPSC frequency of $+19.6\pm4.0\%$ ($n=4$).

3 The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor cyclopiazonic acid (CPA) produced a more robust effect. In 8/10 cells, 25 μM CPA caused a significant increase in mean mIPSC frequency; the mean increase being $+26.0\pm3.0\%$ ($n=8$). Similar results were seen with thapsigargin (1–2 μM), another SERCA pump inhibitor.

4 Ruthenium red (RuR) has been proposed to either act directly on the release machinery or block Ca^{2+} pumps on internal stores. At 10 μM RuR, all cells showed a rapid, large increase in mean mIPSC frequency of $+90.4\pm16.4\%$ ($n=9$). This increase was greater than that seen by agents known to modulate Ca^{2+} stores and was more consistent with a direct action. At this concentration, RuR also occluded the effects of CPA.

5 For all reagents, there were no obvious effects on mean mIPSC amplitude. However, the effects on mIPSC frequency were consistent with a presynaptic action and indicate that Ca^{2+} stores may contribute to spontaneous GABA release onto mouse Purkinje cells.
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Abbreviations: ACSF, artificial cerebro-spinal fluid; ($Ca^{2+}{}_i$), intracellular Ca^{2+}; CPA, cyclopiazonic acid; mIPSCs, miniature inhibitory postsynaptic currents; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; RuR, ruthenium red; TTX, tetrodotoxin; VDCC, voltage-dependent calcium channel

Introduction

Exocytotic neurotransmitter release at presynaptic terminals is driven by a number of Ca^{2+}-dependent interactions between synaptic proteins associated with transmitter vesicles and the plasma membrane (Sudhof, 1995). It is well established that a major mechanism for local rises in intracellular Ca^{2+} ($Ca^{2+}{}_i$) levels is an influx through presynaptic voltage-dependent Ca^{2+} channels (VDCCs) (Augustine, 2001), which associate with the SNARE complex (Sheng *et al*., 1998). Such $Ca^{2+}{}_i$ rises drive action potential-evoked transmitter release. However, transmitter release can occur independently of action potentials and can be measured as so-called 'miniature' postsynaptic events, first described at the frog neuromuscular junction as a basis for the quantal theory of synaptic neurotransmission (Fatt & Katz, 1952). This spontaneous transmitter release was initially proposed to have a trophic influence on subordinate muscle fibres (see Katz, 1966). More recent evidence using *Drosophila* mutants has shown that selective disruption in spontaneous transmitter release leads to defects in clustering of glutamate receptors (Saitoe *et al*., 2001). Tetrodotoxin (TTX)-sensitive spontaneous transmitter release has been shown to occur at many inhibitory synapses in the CNS (Llano *et al*., 1991; Tóth *et al*., 1997; Salin & Prince, 1996; Brickley *et al*., 1996), suggesting that endogenous spontaneous activity is an inherent property of many GABAergic neurons. Large amplitude miniature events occur at many of these synapses and recent evidence suggests that such events in cerebellar Purkinje cells may be due to multivesicular release (Llano *et al*., 2000) and, furthermore, that intracellular calcium stores are of fundamental importance in generating these events.

A number of internal organelles sequester Ca^{2+} in neurons, these include endoplasmic reticulum and mitochondria (Rizzuto, 2001) and such reservoirs afford a potential source of Ca^{2+} for the release machinery. Despite increased knowledge of Ca^{2+} store distribution and molecular composition (Blaustein & Golovina, 2001; Sorrentino & Rizzuto, 2001), our understanding of store contribution to neuronal function remains limited. In the rat cerebellum, immunohistochemical evidence reveals that basket cell terminals innervating Purkinje cells contain ryanodine receptors which functionally

*Author for correspondence; E-mail: g.stephens@ic.ac.uk
[2]Current address: University of Oxford, Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK

modulate miniature inhibitory postsynaptic currents (mIPSCs) (Llano *et al*., 2000). Studies in hippocampal pyramidal cells have also implicated Ca^{2+} stores in contributing to spontaneous excitatory release (Emptage *et al*., 2001) and also to inhibitory GABA release (Savic & Sciancalepore, 1998).

Here, we evaluate the functional contribution of internal Ca^{2+} stores to the generation of GABAergic mIPSCs in Purkinje cells in further detail. In the presence of TTX, mIPSCs persist at a relatively high frequency in these cells and cannot be reduced by subsequent blockage of VDCCs (Stephens *et al*., 2001). Therefore, presynaptic Ca^{2+} stores present in inhibitory terminals onto Purkinje cells represent one potential source of Ca^{2+} for GABA release. We show here that the modulation of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump and, to a lesser extent, the ryanodine receptor could produce increases in mIPSC frequency, whilst the polyvalent cation ruthenium red (RUR) produced more rapid and larger effects more consistent with a direct action on the secretory mechanism. These results support previous studies in central neurons and suggest a potential further role for intracellular Ca^{2+} stores in synaptic function. A preliminary report of some of these findings has appeared in abstract form (Bardo *et al*., 2002).

Methods

Tissue preparation and solutions

Cerebellar slices were prepared according to methods previously described in detail by Southan & Robertson (1998a). Three to four-week old male TO mice (Harlan, U.K.) were killed humanely by cervical dislocation and decapitated in line with U.K. Home Office procedures. The brain was removed and transferred into a chilled, oxygenated sucrose-based artificial cerebro-spinal fluid (ACSF) solution. The cerebellum was dissected out and parasaggital cerebellar slices (300 μm thick) were cut using a Vibroslice™ (Campden Instruments, U.K.). Slices were transferred into oxygenated standard ACSF solution at 37°C for 1 h and then maintained at room temperature (20–24°C). The standard ACSF contained (in mM): NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 2.5, $MgSO_4$ 2, $CaCl_2$ 2, D-glucose 10, maintained at pH 7.3 by bubbling with 95% O_2/5% CO_2. The sucrose-based ACSF solution used for dissection and slicing was identical with the exception that NaCl was replaced by iso-osmotic sucrose (74.5 g l^{-1}).

Electrophysiological recording

Slices were placed in a recording chamber and perfused at 2–4 ml min^{-1} with standard ACSF bubbled with 95% O_2/5% CO_2. Individual neurons were visualized through a 63× water immersion lens using an upright Olympus BX50WI microscope (Olympus, Tokyo, Japan). Purkinje cell somata were readily identified by their characteristic morphology and arrangement within the cerebellar folia. Whole-cell recordings were performed using an EPC-9 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany), controlled by Pulse software (HEKA) with a Macintosh G4 computer. Electrodes were made from borosilicate glass (GC150-F10, Harvard Apparatus, Kent, U.K.) and, when filled with an intracellular solution containing (in mM): CsCl 140, $MgCl_2$ 1, $CaCl_2$, EGTA 10, HEPES 10, pH 7.3, had resistances between 3–5 MΩ. Series resistance was typically 5–9 MΩ and was monitored and compensated by 65–85% throughout. Data was sampled at 7 kHz and filtered at one third of the sampling frequency. mIPSCs were identified as rapidly-activating inward currents at a holding potential of −70 mV. Events were collected over 2 s sweeps using automatic peak detection with all individual events confirmed by eye. Data analysis was carried out using Pulsefit (HEKA), Axograph (Axon Instruments) and Excel (Microsoft) software.

Cumulative frequency plots were constructed by integrating a probability density function histogram with a bin width of 2 pA (mIPSC amplitude plots) or 5 ms (mIPSC inter-event interval plots). Data are presented as means ± s.e.m., where (n) = number of cells, statistical significance was determined using a paired or unpaired Student's t-test or Kolmogorov–Smirov (KS) tests as appropriate.

Pharmacology

The following reagents were used: ryanodine, cyclopiazonic acid (CPA), thapsigargin, ruthenium red (all Sigma, U.K.). All drugs were made up as concentrated (1000×) stock solutions in distilled water (or DMSO for CPA and thapsigargin), aliquoted and stored at −20°C; aliquots were thawed and dissolved in oxygenated ACSF immediately prior to use. Reagents were applied in the presence of the Na^+ channel antagonist TTX (1 μM, Alomone, Israel) to eliminate any contribution from action potential-dependent IPSCs.

Results

Isolation of miniature inhibitory postsynaptic currents (mIPSCs)

In the present study we investigated the potential contribution of Ca^{2+} stores to the generation of mIPSCs in mouse cerebellar Purkinje cells. In these cells, mIPSCs are mediated by GABA acting on $GABA_A$ receptors, as shown by sensitivity to bicuculline (e.g. Southan & Robertson, 1998b). We define spontaneous GABA release as the action potential-independent events that persist in the presence of TTX. Purkinje cells are ideally suited to these studies, as a relatively high frequency of GABA release is maintained in TTX (Llano *et al*., 1991; Southan & Robertson, 1998b; Stephens *et al*., 2001). Application of 1 μM TTX caused clear reductions in mean IPSC amplitude from −75.8 ± 5.6 pA to −46.8 ± 3.9 pA (n = 29, P < 0.001), and mean IPSC frequency from 12.3 ± 1.5 Hz to 3.6 ± 0.4 Hz (n = 29, P < 0.001) in these cells (data not shown). Percentage reduction in these parameters were of very similar magnitude to values reported previously (Stephens *et al*., 2001); TTX effects were not accompanied by any consistent effect on the time course of decay of events or changes in holding current. Prior to application of reagents a stable base line was established in TTX; no spontaneous increases in mIPSC frequency (e.g. Diana *et al*., 2002) were observed prior to, or following, drug application.

Effects of ryanodine on mIPSCs

Having established conditions to isolate stable mIPSCs, we investigated the actions of a number of agents reported to release Ca^{2+} from internal stores, to determine if these could elicit spontaneous GABA release in the absence of a contribution from VDCCs. Firstly, we investigated the effects of the plant alkaloid ryanodine, which acts as an inhibitor of endoplasmic reticulum ryanodine-sensitive Ca^{2+} release channels (ryanodine receptors) at low micromolar concentrations (see Sutko *et al*., 1997) (Figure 1). Ryanodine effects were confined to a subpopulation of cells tested. Bath application of 10 μM ryanodine for 10–15 mins induced a significant increase in mean mIPSC frequency in 4/12 cells tested ($P<0.05$, KS test); the mean increase was $+19.6\pm4.0\%$ ($n=4$). In the remaining eight cells no significant changes in mIPSC frequency were seen. Figure 1A–C illustrates an example of a ryanodine-sensitive cell. Onset of ryanodine action occurred with a slow time course, taking around 10 min to reach full effect (Figure 1B), and, as in other responsive cells, showed little or no reversibility in washes up to 30 min. In responsive cells, the increase in mIPSC frequency can be illustrated by a cumulative frequency plot of mean mIPSC inter-event intervals (Figure 1C). Overall, no consistent effects were seen on mean mIPSC amplitude (Figure 1D): control mean mIPSC amplitude (-55.0 ± 6.9 pA $n=12$) was unaffected by 10 μM ryanodine (-55.0 ± 6.2 pA $n=12$, $P=0.986$). Similarly, in the four responsive cells, control mean mIPSC amplitude (-50.4 ± 7.6 pA $n=4$) was also unaffected by 10 μM ryanodine (-53.5 ± 10.2 pA $n=4$, $P=0.493$). The effects of ryanodine on inter-event interval (mIPSC frequency), together with the lack of any obvious effects on mIPSC amplitude, are consistent with a presynaptic site of action causing an increase in the release probability of vesicles, and also suggests that the sensitivity of postsynaptic $GABA_A$ receptors was not affected.

Effects of cyclopiazonic acid on mIPSCs

We next examined the effects of the SERCA pump inhibitor cyclopiazonic acid (CPA) on mIPSCs. Bath application of 25 μM CPA produced a more consistent effect than that seen with ryanodine (Figure 2). Application of CPA for 10–15 mins was accompanied by an increase in mean mIPSC frequency in 8/10 cells tested ($P<0.05$, KS test); an examplar cell is illustrated in Figure 2A,B. Onset of action was typically slow, taking around 10 min to reach full effect, and showed little reversibility in up to 30 mins of washing (Figure 2B). In responsive cells, 25 μM CPA caused a significant increase in mean mIPSC frequency of $+26.0\pm3.0\%$ ($n=8$). The pooled ($n=8$) cumulative frequency distribution of mean mIPSC inter-event intervals illustrates this CPA-induced increase in frequency (Figure 2C). In contrast, control mean mIPSC amplitude in responsive cells (-35.2 ± 4.5 pA $n=8$) was unaffected by 25 μM CPA (-36.3 ± 3.9 pA $n=8$,

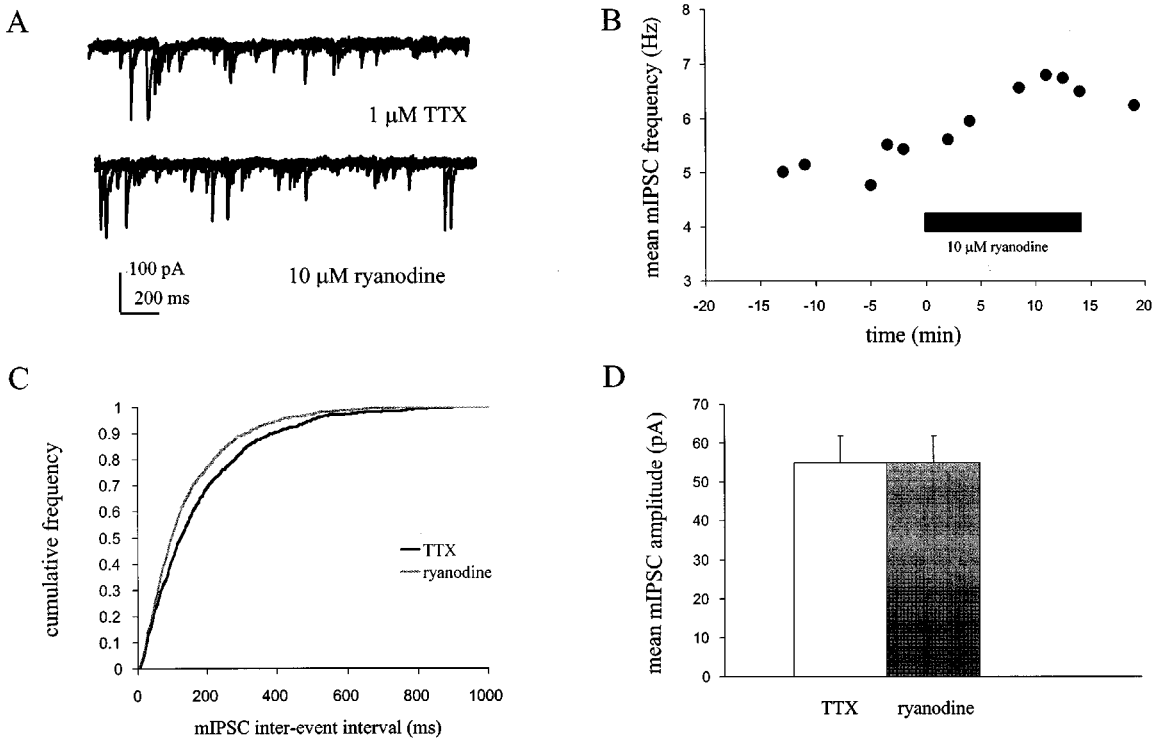

Figure 1 Effects of ryanodine on Purkinje cell mIPSCs. (A) Example of 10 μM ryanodine-induced increase in mIPSC frequency. Raw data are three consecutive 2 s sweeps superimposed in either TTX control or steady-state ryanodine conditions; $V_H=-70$ mV. (B) Time course of action of ryanodine (black bar) on mean mIPSC frequency for example cell shown in A. Ryanodine caused a slow onset increase in frequency. (C) Cumulative frequency plot for the effects of ryanodine on control mIPSC inter-event interval of example cell shown in A; bin width 5 ms. Ryanodine caused a significant shortening of the mIPSC inter-event interval ($P<0.05$, KS test) in this cell. (D) Effects of ryanodine on mean mIPSC amplitude. Ryanodine (10 μM, $n=12$) had no significant effect on control mean mIPSC amplitude ($n=12$, white bar).

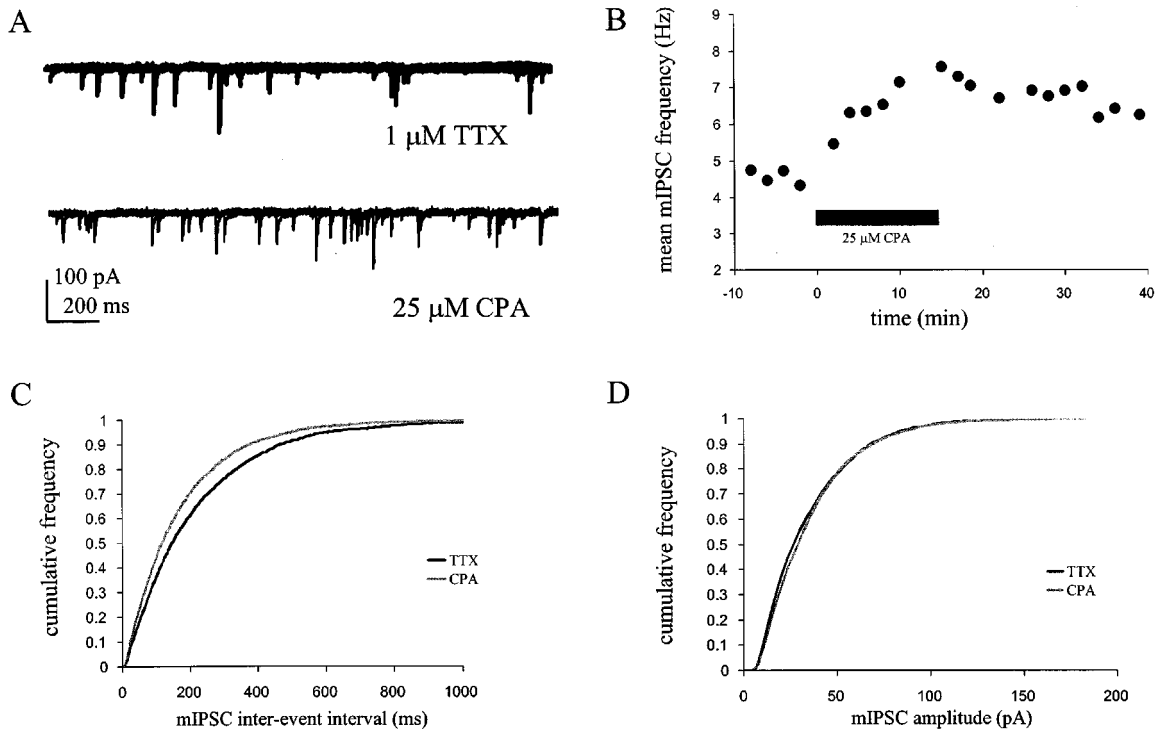

Figure 2 Effects of cyclopiazonic acid (CPA) on Purkinje cell mIPSCs. (A) Example of 25 μM CPA-induced increase in mIPSC frequency. Raw data are three consecutive 2 s sweeps superimposed in either TTX control or steady-state CPA conditions; $V_H = -70$ mV. (B) Time course of action of CPA (black bar) on mean mIPSC frequency for example cell shown in A. CPA caused a slow onset increase in frequency. (C) Pooled cumulative frequency plot ($n=8$) for CPA effects on control mIPSC inter-event interval; bin width 5 ms. CPA caused a significant shortening of the mIPSC inter-event interval ($P<0.05$, KS test). (D) Pooled cumulative frequency plot ($n=8$) for CPA effects on control mIPSC amplitude; bin width 2 pA. CPA had no significant effect on mIPSC amplitude.

$P=0.646$), as illustrated in a pooled ($n=8$) cumulative frequency distribution which showed no clear differences before, or after CPA application (Figure 2D). The increase in mIPSC frequency induced by CPA is consistent with a presynaptic site of action.

We also tested the effects of thapsigargin, another SERCA pump inhibitor. Pooled data from experiments with bath application of 1 or 2 μM thapsigargin for 10–15 mins produced similar results to those seen due to SERCA pump inhibition by CPA (data not shown). In 6/7 cells, thapsigargin (1–2 μM) increased mean mIPSC frequency ($P<0.05$, KS test); in responsive cells the mean increase was $+38.6\pm11.0\%$ ($n=6$). Again, control mean mIPSC amplitude in these cells (-60.2 ± 14.1 pA $n=6$) was unaffected by thapsigargin (-58.5 ± 15.4 pA $n=6$, $P=0.565$).

Effects of ruthenium red on mIPSCs

Studies at the frog neuromuscular junction showed originally that external application of ruthenium red (RuR) increased miniature events (Alnaes & Rahamimoff, 1975). RuR has been proposed to act intracellularly to block the Ca^{2+} uniporter on mitochondrial Ca^{2+} stores (Moore, 1971) and/or the ryanodine receptor (Nagasaki & Fleischer, 1989). In apparent contrast, many reports indicate that RuR shows little membrane permeability and may act directly on the synaptic vesicle release machinery (Trudeau *et al*., 1996a). Therefore, we were interested in examining the effects on RuR on mouse Purkinje cells. Bath application of 10 μM RuR for 10–15 mins induced large increase in mean mIPSC frequency in all cells tested ($n=9$; $P<0.05$, KS test) (Figure 3). Figure 3A,B show an examplar cell; typically the time course of action was relatively rapid, reaching a peak between 4–6 mins and showing limited reversibility over 20 mins wash. In these cells, 10 μM RuR caused a major increase in mean mIPSC frequency of $+90.4\pm16.4\%$ ($n=9$). The pooled ($n=9$) cumulative frequency distribution of mean mIPSC inter-event interval illustrates this large RuR-induced increase (Figure 3C). In contrast, control mean mIPSC amplitude (-48.4 ± 4.4 pA $n=9$) was unaffected by RuR treatment (-52.9 ± 5.5 pA $n=9$, $P=0.761$), with a pooled ($n=9$) cumulative frequency distribution revealing no clear differences before or after RuR application (Figure 3D). The increase in mIPSC frequency induced by RuR is consistent with a presynaptic site of action and the relatively rapid time course is more suggestive of the hypothesis of a direct (extracellular) action.

Effects of reagents on the frequency of small, intermediate and large amplitude mIPSCs

We next looked for any differences between the action of RuR and agents that modulate Ca^{2+} stores (Figure 4). In summary of the data above, effects were confined to increases in mIPSC frequency, with no consistent effects on mIPSC amplitude, suggesting that all reagents acted presynaptically. At the concentrations used, RuR-induced increases in mIPSC frequency were significantly larger in comparison to responses to ryanodine ($P<0.01$), CPA ($P<0.001$) and thapsigargin

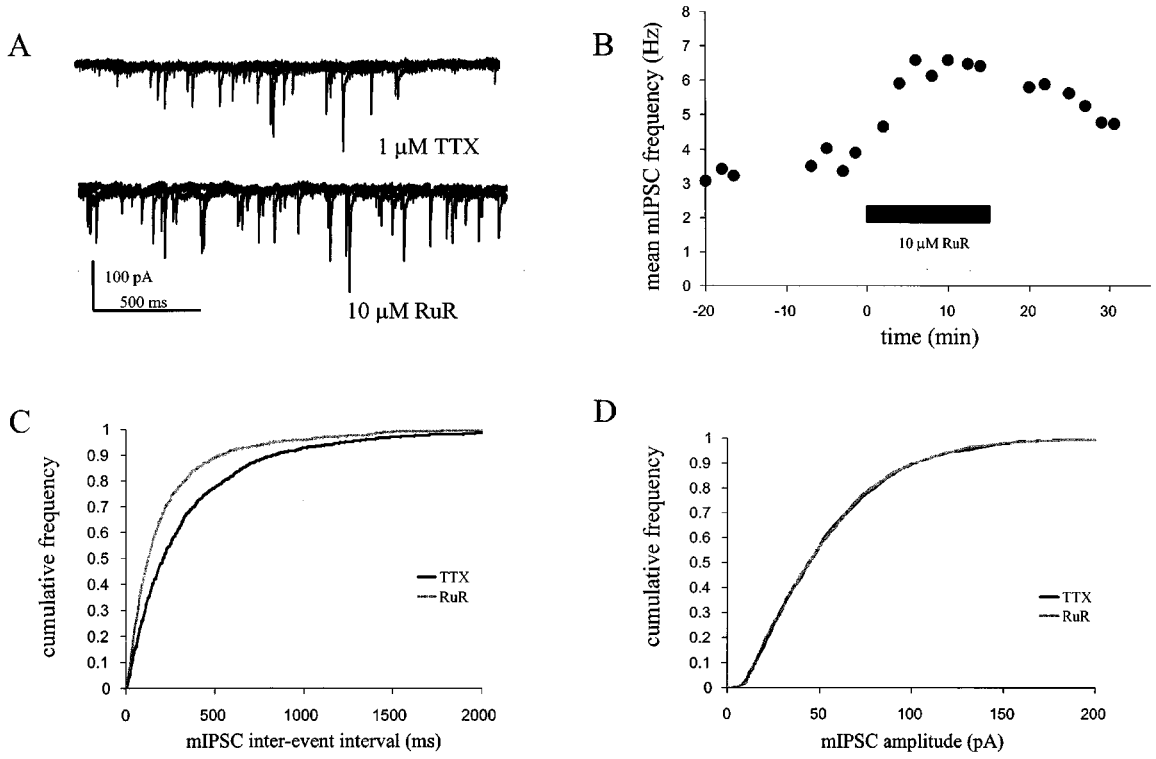

Figure 3 Effects of ruthenium red (RuR) on Purkinje cell mIPSCs. (A) Example of 10 μM RuR-induced increase in mIPSC frequency. Raw data are three consecutive 2 s sweeps superimposed in either TTX control or steady-state RuR conditions; $V_H = -70$ mV. (B) Time course of action of RuR (black bar) on mean mIPSC frequency for example cell shown in A. RuR caused a relatively rapid onset increase in frequency. (C) Pooled cumulative frequency plot ($n=9$) for RuR effects on control mIPSC inter-event interval; bin width 5 ms. RuR caused a significant shortening of the mIPSC inter-event interval ($P<0.05$, KS test). (D) Pooled cumulative frequency plot ($n=9$) for RuR effects on control mIPSC amplitude; bin width 2 pA. RuR had no significant effect on mIPSC amplitude.

($P<0.05$) (Figure 4A). In contrast, there were no differences in the magnitude of effects between the cells responsive to modulation of ryanodine receptors and SERCA pumps. The mIPSC population represents a continuum of responses, including some unusually large amplitude mIPSCs ('maximinis') which have been proposed to be regulated by Ca^{2+} stores (Llano *et al*., 2000). We analysed our results in more detail to determine if any selective changes amongst the different mIPSC amplitude populations occurred as a result of either the excitatory action of RuR or by modulators of Ca^{2+} stores (Figure 4B). We defined large mIPSCs as >50 pA and divided remaining events into small (0–25 pA) and intermediate (26–50 pA) bins. RuR (10 μM) ($n=9$) caused increases in mIPSC frequency for each population of small ($+66.0\pm21.6\%$), intermediate ($+73.0\pm14.4\%$) and large ($+95.9\pm24.9\%$) amplitude mIPSC events (Figure 4Bi). These divisions were not statistically different from one another, with the large mIPSC bin being somewhat skewed by one cell that showed an atypical large increase in >50 pA events. Similar results were obtained in the cells that were responsive (based on a significant increase in mIPSC frequency at a level of $P<0.05$, KS test) to the application of ryanodine ($n=4$), CPA ($n=8$) and thapsigargin ($n=6$). The increases in mIPSC frequency induced by agents that modulate Ca^{2+} stores were combined ($+27.7\pm3.4\%$, $n=18$) and compared between mIPSC populations (Figure 4Bii). Again no selective increases in mIPSC frequency between small ($+25.5\pm27.9\%$), intermediate ($+22.8\pm8.7\%$) and large ($+25.5\pm16.8\%$) amplitude mIPSC events were seen. Overall these results show that, at concentrations used, RuR caused larger increases in mIPSC frequency than agents which modulated stores, and that increases in spontaneous GABA release were not due to a selective enhancement of a specific population of responses by either treatment.

Finally, we determined the effects of RuR pre-treatment on the actions of CPA, the most robust agent on intracellular stores (Figure 5). In the continued presence of RuR, CPA was now no longer able to induce the increase in mIPSC frequency seen previously (Figure 2). In 5/5 cells, addition of 10 μM RuR caused a robust increase in mIPSC frequency (mean increase $= +122\pm34\%$ $n=5$); again, control mIPSC amplitude (-39.4 ± 8.4 pA $n=5$) was not significantly changed by 10 μM RuR (-44.6 ± 11.1 pA $n=5$, $P=0.299$), in agreement with previous data (Figure 3). The subsequent application of 25 μM CPA was now without effect on mIPSC frequency in all five cells tested. These data are illustrated in an exemplar cell time course (Figure 5A) and summarized by a pooled ($n=5$) cumulative frequency plot for mIPSC inter-event interval (Figure 5B). CPA (25 μM) was again without effect on mIPSC amplitude (-44.6 ± 11.2 pA $n=5$, $P=0.382$), as shown in a pooled ($n=5$) cumulative frequency plot (Figure 5C). Therefore, these data show that, at the concentrations used, RuR effectively occludes the action of CPA, an agent that acts on internal stores and shown to be capable of inducing GABA release onto Purkinje cells.

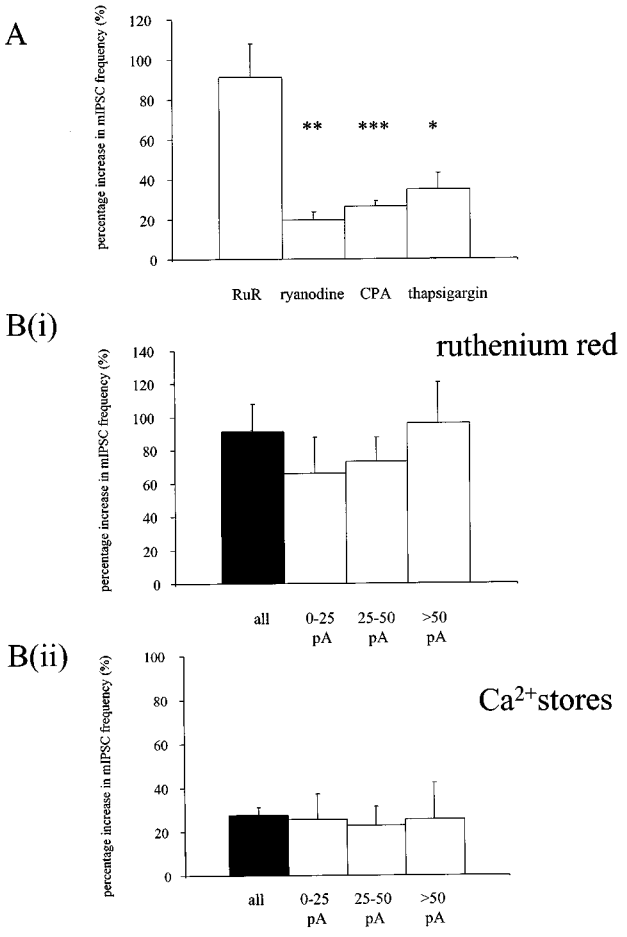

Figure 4 Effects of reagents on the frequency of small, intermediate and large amplitude mIPSCs. (A) Effects of different treatments on percentage increase in control mIPSC frequency. RuR (10 μM, $n=9$) caused a significantly greater increase in mean mIPSC frequency than 10 μM ryanodine ($n=4$, $**=P<0.01$), 25 μM CPA ($n=8$, $***=P<0.001$) and 1–2 μM thapsigargin ($n=6$, $*=P<0.05$). (Bi) Effect of 10 μM RuR ($n=9$) on the frequency of small- (0–25 pA), intermediate- (26–50 pA) and large- (>50 pA) amplitude mIPSCs. No clear differences in RuR-induced increases were seen between each group. (Bii) Effect of modulation of Ca^{2+} stores ($n=18$, group data from 10 μM ryanodine, $n=4$; 25 μM CPA, $n=8$; 1–2 μM thapsigargin, $n=6$) on the frequency of small- (0–25 pA), intermediate- (26–50 pA) and large- (>50 pA) amplitude mIPSCs. No clear differences in Ca^{2+} store modulator-induced increases were seen between each group.

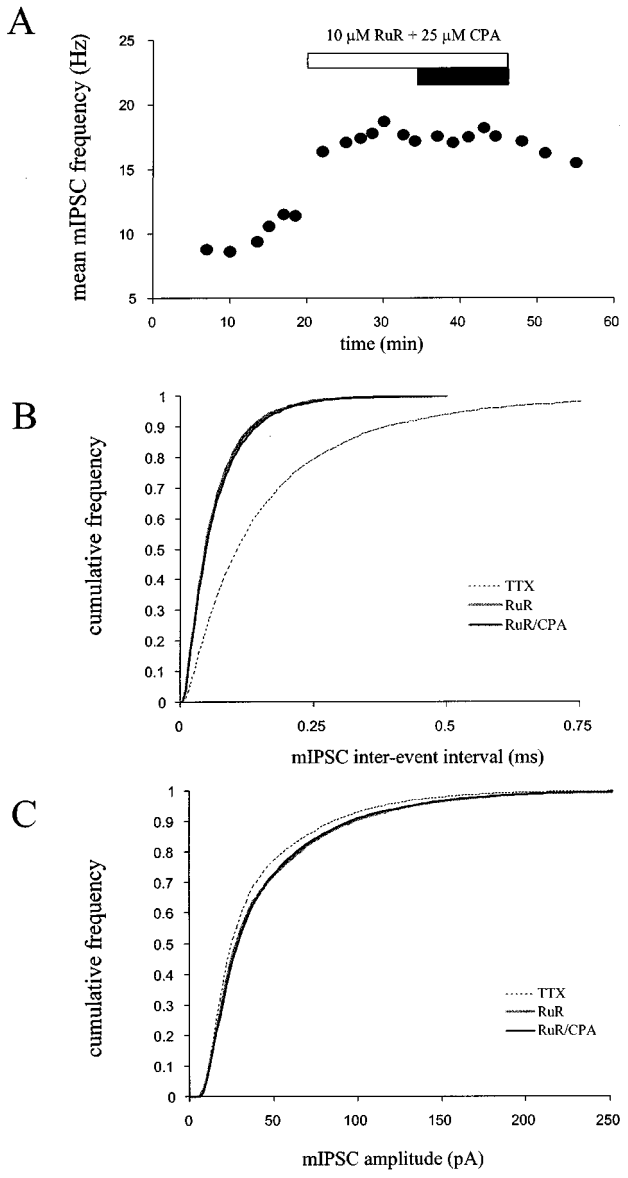

Figure 5 Effects of CPA on Purkinje cell mIPSCs in the presence of RuR. (A) Example of occlusion of effects of 25 μM CPA by 10 μM RuR. Time course of action of RuR (white bar) and subsequent application of CPA (black bar) on mean mIPSC frequency. RuR caused a relatively rapid onset increase in frequency, CPA had no further effects. (B) Pooled cumulative frequency plot ($n=5$) for RuR effects and RuR/CPA on control mIPSC inter-event interval; bin width 5 ms. RuR caused a significant shortening of the control mIPSc inter-event interval ($P<0.05$, KS test), but CPA had no further effects. (D) Pooled cumulative frequency plot ($n=9$) for RuR effects and RuR/CPA on control mIPSC amplitude; bin width 2 pA. Neither RuR nor RuR/CPA had any significant effect on mIPSC amplitude.

Discussion

Contribution of Ca^{2+} stores to GABA release onto Purkinje cells

The present study demonstrates that internal organelles which store Ca^{2+} can make a functional contribution to spontaneous transmitter release in central neurons. In mouse Purkinje cells, mIPSCs arise due to the spontaneous, action potential-independent release of GABA from presynaptic terminals (e.g. Southan & Robertson, 1998b); however, the mechanisms underlying the generation of mIPSCs are largely unknown. We have shown previously that action potential-evoked release is mediated by VDCCs, and also that miniature events are not affected by block of VDCCs (Stephens *et al.*, 2001). In this study, Ca^{2+} influx into presynaptic terminals *via* the major VDCC pathway is eliminated by TTX treatment, allowing the effects of manipulating Ca^{2+} stores to be studied in isolation. Under these conditions, we demonstrate that modulation of ryanodine receptors and SERCA pumps, can lead to increases in the frequency of spontaneous GABA release. Purkinje cells are subject to tonic inhibition from GABAergic

interneurons which modulates the shape and propagation of action potentials (Hausser & Clark, 1997) and spontaneous transmitter release has also been implicated in receptor clustering at synapses (Saitoe *et al*., 2001), modulation of GABA release by Ca^{2+} stores could therefore have important physiological consequences.

Activation of ryanodine receptors by a low concentration of ryanodine (10 μM) produced excitatory effects confined to a subpopulation of Purkinje cells. This is in agreement with discriminatory effects seen in other studies, where similar concentrations of ryanodine caused an increase in mIPSC frequency in 4/10 rat Purkinje cells (Llano *et al*., 2000) and 2/9 hippocampal pyramidal neurons tested (Savic & Sciancalepore, 1998). There are a number of possible explanations for the lack of a consistent effect of ryanodine. The efficacy of ryanodine may reflect a heterogeneity in the functional status of the Ca^{2+} store, which may be only partially full under resting conditions (Garaschuk *et al*., 1997). Sequestration of Ca^{2+} is a dynamic process and degree of Ca^{2+} store loading may be a crucial determinant for a contribution to a number of neuronal functions (see Berridge, 1998). A further complication may arise from the mechanism of action of ryanodine. Whilst low/sub micromolar concentrations of ryanodine lock receptors in a sub-conductance state, higher micromolar concentrations block the receptor (Sutko *et al*., 1997). Functionally, application of higher ryanodine concentrations (100 μM) to brain slices has been shown to cause reductions in spontaneous GABA release onto Purkinje cells (Llano *et al*., 2000). The discrepancy in effective concentration is illustrated in a study by Emptage *et al*. (2001) in which 20–30 μM ryanodine acted as an antagonist at ryanodine receptors in hippocampal pyramidal cells in organotypic brain slices. A further possibility is that the heterogeneity of ryanodine effects may be due to regional differences in receptor and channel distribution within the cerebellum, see for example Stephens *et al*. (2001) and references therein. We also tested the effects of caffeine, another commonly used ryanodine receptor agonist. However, bath application of 10 mM caffeine caused a rapid and profound reduction of both mean mIPSC amplitude and frequency (data not shown). The effect of caffeine on mIPSC amplitude is consistent with a postsynaptic action, most likely due to a direct effect on $GABA_A$ receptors (Savic & Sciancalepore, 1998), and therefore caffeine was not used further here. In light of this and the heterogeneity in ryanodine response, we focussed on other modulators of Ca^{2+} stores.

The effects of blocking the SERCA pump by CPA and thapsigargin proved a more reliable method of eliciting effects on spontaneous GABA release, causing increases in mIPSC frequency in the vast majority of cells tested. The increased population of responsive cells in comparison to that seen with ryanodine, indicates that heterogeneity in the status of the Ca^{2+} store (see above) may not be such an important determinant of action. This differential effect may simply reflect that SERCA pump inhibition is a more reliable method of generating increases in $Ca^{2+}{}_i$ than activation of ryanodine receptors. The SERCA pump functions to maintain a large Ca^{2+} concentration gradient between the lumen of the store (10–100 μM) and the cytosol (100–300 nM) (Mattson *et al*., 2000). Dissipation of this gradient by SERCA pump inhibitors leads to an actual depletion of Ca^{2+} stores (Seidler *et al*., 1989), in contrast to the concentration-dependent action of ryanodine. Alternatively, SERCA pumps may be more closely localized to GABA release. Although both ryanodine receptors and SERCA pumps are widely expressed in the endoplasmic reticulum (Blaustein & Golovina, 2001), which in turn may come into close association with the secretory apparatus in neurons (Westrum & Gray, 1986), alternative sites of action may also be important. An intriguing and germane suggestion is that the membrane of synaptic vesicle themselves may contain SERCA pumps (Castonguay & Robitaille, 2001), any Ca^{2+} generated here would be ideally localized to contribute to release.

Potential sites and mechanisms of action of agents that modulate Ca^{2+} stores

The mechanisms of action of ryanodine, CPA and thapsigargin on Ca^{2+} stores are well established. In the present study, perfusion of cerebellar slices with nominally Ca^{2+}-free ACSF supplemented with 2 mM $MgCl_2$ and 200 μM EGTA resulted in a major deterioration and loss of the whole-cell recording configuration. In addition, pretreatment of slices with 200 μM BAPTA-AM failed to chelate intracellular calcium, as 25 μM CPA was still able to cause an increase in mean mIPSC frequency of $+23.5 \pm 5.6\%$ in 4/5 cells tested (data not shown). However, it has been demonstrated recently using imaging techniques, that both ryanodine (Llano *et al*., 2000) and CPA (Emptage *et al*., 2001) do increase calcium transients in brain slices and, importantly, that such effects lead to increases in spontaneous transmitter release.

For the reagents that modulated Ca^{2+} stores, effects on mIPSC frequency were not accompanied by any clear changes in mIPSC amplitude; this suggests that the sensitivity of postsynaptic $GABA_A$ receptors was not affected in these experiments. In fact, these findings are consistent with a presynaptic site of action, whereby manipulation of internal stores in presynaptic terminals onto Purkinje cells can elicit an increase in the release probability of vesicles. Postsynaptic rises in $Ca^{2+}{}_i$ in Purkinje cells have been widely implicated in the process of depolarization-induced suppression of inhibition (DSI) (Llano *et al*., 1991). Cerebellar DSI is due to Purkinje cell depolarization causing release of a diffusable retrograde messenger (most likely an endogenous cannabinoid), which acts presynaptically to modulate GABA release (Kreitzer & Regehr, 2001; Diana *et al*., 2002). DSI also modulates spontaneous GABA release, but, importantly, is associated with a decrease in mIPSC frequency (Diana *et al*., 2002) and cannot explain the results presented here.

Immunohistochemical studies with a consensus anti-ryanodine receptor antibody have shown that synaptic terminals of basket cell interneurons in rat cerebellum contain ryanodine receptors (Llano *et al*., 2000). As such, basket cell terminals are a potential site of action of ryanodine receptor and SERCA pump inhibitors in this study. Within presynaptic terminals ryanodine receptors and SERCA pumps are prominent on endoplasmic reticulum (and SERCA pumps may also be present in synaptic vesicle membranes). In addition, ryanodine receptors have also been identified in mitochondria (Beutner *et al*., 2001); it will be of interest to determine if mitochondrial Ca^{2+} stores in neurons can contribute to spontaneous transmitter release.

Effect of ruthenium red on GABA release onto Purkinje cells

Bath application of the polyvalent cation RuR caused relatively large, rapid increases in mIPSC frequency in all Purkinje cells tested. Together with the lack of consistent effects on mIPSC amplitude, this again suggests a presynaptic site of action. External application of RuR was shown originally to increase miniature events at the frog neuromuscular junction (Alnaes & Rahamimoff, 1975). Although RuR is widely used as an inhibitor of the mitochondrial Ca^{2+}-uniporter, more recent reports suggest it must be dialysed or injected into cells to work by this mechanism (Tang & Zucker, 1997; Hernandez-Guijo *et al*., 2001). RuR has also been shown to increase transmitter release by a direct, Ca^{2+}-independent effect on the secretory apparatus (Trudeau *et al*., 1996a); this action was sensitive to tetanus toxin, indicating that the mechanism relied on correct formation of the SNARE complex prior to vesicular release (Trudeau *et al*., 1996b). Here, the time course and magnitude of action were more consistent with an interaction with a site associated with the external cell membrane. A similar mechanism has been proposed for RuR effects on spontaneous GABA release onto hippocampal pyramidal cells (Sciancalepore *et al*., 1998) and cells dissociated from basolateral amygdala nuclei (Koyama *et al*., 1999).

No selective effects on small or large amplitude mIPSCs were shown by either RuR or agents known to modulate Ca^{2+} stores. Presynaptic Ca^{2+} stores have been suggested to underlie large amplitude mIPSCs in rat cerebellar Purkinje cells (Llano *et al*., 2000); these 'maximinis' were proposed to be due to multivesicular GABA release and were selectively reduced by blocking ryanodine receptors using 100 μM ryanodine. However, in agreement with the present study, at lower, 'excitatory' ryanodine concentrations (10 μM), both smaller events and maximinis were increased to similar extents; this was possibly due to a saturation of postsynaptic $GABA_A$ receptors (see Llano *et al*., 2000). A clear distinction between actions of RuR *versus* reagents that act intracellularly on Ca^{2+} stores was in the magnitude of the effect on frequency of GABA release. If we assume a common endpoint (presynaptic GABA release from vesicles), these data suggest that RuR may act at a site further downstream to internal stores. The occlusion of CPA effects by RuR is also consistent with this proposal and a direct action of RuR on the synaptic machinery would fulfil these criteria.

Contribution of Ca^{2+} stores to neuronal function

In addition to the well known functions of internal organelles in sequestering and buffering $Ca^{2+}{}_i$, recent studies have demonstrated that Ca^{2+} stores may make important contributions to many aspects of neuronal signalling, including maturation, plasticity and cell death (reviewed by Rizzuto, 2001). The present work extends our physiological knowledge of these functional contributions, showing that modulation of Ca^{2+} stores can elicit spontaneous neurotransmitter release in the absence of Ca^{2+} influx through VDCCs. It adds to a number of recent studies demonstrating a role of internal Ca^{2+} stores in spontaneous inhibitory and excitatory transmitter release (Savic & Sciancalepore, 1998; Llano *et al*., 2000; Emptage *et al*., 2001). A similar role for Ca^{2+} stores in the spontaneous release of excitatory transmitter was not observed in CA3 pyramidal neurons (Henze *et al*., 2002) as ryanodine, CPA and thapsigargin all had only modest effects on 'giant' mEPSC frequency. Interestingly however, the ryanodine receptor agonist caffeine did produce robust increases in frequency to levels similar to those seen with the secretagogue α-latrotoxin. It is unclear if this reflects differences between individual synapses; for instance, the mossy fibre terminals that supply CA3 pyramidal cells contain specialized large, clear vesicles which have a different distribution within the active zone than smaller vesicles (Henze *et al*., 2002). It will be of interest in the future to determine the precise location of the Ca^{2+} stores contributing to spontaneous GABA release onto mouse Purkinje cells demonstrated in this study, both in terms of identification of the presynaptic cell type and, also, the relative contribution from different Ca^{2+}-sequestering internal organelles within presynaptic terminals.

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