# Inositol Trisphosphate and Ryanodine Receptors Share a Common Functional $Ca^{2+}$ Pool in Cerebellar Purkinje Neurons

Kamran Khodakhah and Clay M. Armstrong
Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6085 USA

ABSTRACT Changes in the intracellular free calcium concentration ($[Ca^{2+}]_i$) control many important processes in excitable and nonexcitable cells. In cerebellar Purkinje neurons, increases in $[Ca^{2+}]_i$ modulate excitability by turning on calcium-activated potassium and chloride conductances, and modifying the synaptic efficacy of inhibitory and excitatory inputs to the cell. Calcium release from the intracellular stores plays an important role in the regulation of $[Ca^{2+}]_i$. Purkinje neurons contain both inositol trisphosphate ($InsP_3$) and ryanodine (Ry) receptors. With the exception of the dendritic spines, where only $InsP_3$ receptors are found, $InsP_3$ and Ry receptors are present in the entire cell. The distribution of the two calcium release channels, however, is not uniform, and it has been suggested that $InsP_3$ and Ry receptors use separate $Ca^{2+}$ pools. The functional properties of $InsP_3$ and Ry $Ca^{2+}$ pools were investigated by flash photolysis and single-cell microspectrofluorimetry. It was found that depletion of ryanodine-sensitive $Ca^{2+}$ stores renders $InsP_3$ incapable of releasing more $Ca^{2+}$ from the stores. Abolishing calcium-induced calcium release by blocking ryanodine receptors with ruthenium red did not have a significant effect on $InsP_3$-evoked $Ca^{2+}$ release. It is concluded that $InsP_3$ receptors use the same functional $Ca^{2+}$ pool as that utilized by Ry receptors in Purkinje neurons.

## INTRODUCTION

Calcium release from intracellular stores plays an important role in controlling processes such as contraction, secretion, cell growth, and fertilization (Berridge, 1993a,b, 1995). In the central nervous system, calcium release from intracellular stores is thought to modulate membrane excitability and synaptic plasticity, in addition to neuronal growth (Henzi and MacDermott, 1992; Furuchi and Mikoshiba, 1995).

Two distinct calcium release channels, namely the ryanodine (Ry) and inositol trisphosphate ($InsP_3$) receptors, regulate release of calcium from intracellular stores. Purkinje neurons contain the highest density of $InsP_3$ binding sites in the central nervous system (Ross et al., 1989; Sharp et al., 1993); these are almost exclusively the type 1 variant (DeSmedt et al., 1994). These calcium release channels are found in the soma, axon, dendrites, and dendritic spines of these cells (Ellisman et al., 1990; Walton et al., 1991; Takei et al., 1992; Sharp et al., 1993). Purkinje neurons also express ryanodine receptors (Ellisman et al., 1990; Kuwajima et al. 1992) of the cardiac (McPherson et al., 1991; Lai et al., 1992) and skeletal (Kuwajima et al., 1992) types, although the distribution of Ry-Rs is not identical to that of $InsP_3$ receptors (Walton et al., 1991; Sharp et al., 1993). A clear difference in the localization of these two calcium release channels is that $InsP_3$, but not Ry receptors, is found in the dendritic spines of Purkinje neurons (Ellisman et al., 1990; Walton et al., 1991; Sharp et al., 1993).

There is good evidence that changes in the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) control many processes in the Purkinje neurons. A rise in $[Ca^{2+}]_i$ directly modulates excitability of these neurons by turning on calcium-activated potassium (Khodakhah and Ogden, 1992, 1995) and probably chloride (Llano et al., 1991a) conductances. Changes in the $[Ca^{2+}]_i$ are shown to be required for the modulation of the strength of excitatory and inhibitory synaptic inputs to a Purkinje neuron (Sakurai, 1990; Konnerth et al., 1992; Llano et al., 1991b; Vincent et al., 1992; Kano et al., 1992). Increase in $[Ca^{2+}]_i$ by calcium release from the intracellular stores has been demonstrated by direct activation of $InsP_3$ receptors (Khodakhah and Ogden, 1992, 1995), activation of the glutamate metabotropic receptors coupled to $InsP_3$ receptors (Llano et al., 1991a: Vranesic et al., 1991), calcium-induced calcium-release (CICR) (Llano et al., 1994; Kano et al., 1995), and application of caffeine (Brorson et al., 1991; Kano et al., 1995). Furthermore, it has been proposed that calcium release from the stores is important for the induction of long-term depression (LTD) in Purkinje neurons (Daniel et al., 1992; Berridge, 1993b; Kasono and Hirano, 1995; Kohda et al., 1995; Khodakhah, 1996; Khodakhah and Armstrong, manuscript submitted for publication).

Given the importance of intracellular calcium release in the modulation of excitability and physiology of Purkinje neurons, it is critical to understand the functional properties of the calcium stores present in these cells. It is of particular interest to establish whether both $InsP_3$ and Ry receptors mobilize calcium from a common calcium pool, or alternatively, given the difference in their cellular distributions, whether they each have a separate $Ca^{2+}$ pool. Present data from light and electron microscopic (Ellisman et al., 1990;

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Address reprint requests to Dr. Kamran Khodakhah, Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6085. Tel.: 215-898-7816; Fax: 215-573-5851; E-mail: khodakhah@mail.med.upenn.edu.



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Walton et al., 1991; Takei et al., 1992; Sharp et al., 1993; Nori et al., 1993) and subfractionation (Volpe et al., 1991; Nori et al., 1993) studies have provided evidence in favor of the existence of heterogeneous $Ca^{2+}$ stores. There are, however, no direct functional studies on the properties of the $Ca^{2+}$ stores in Purkinje neurons. By combining flash photolytic release of $InsP_3$ with microspectrofluorimetry in voltage-clamped Purkinje cells, we attempted to establish whether $InsP_3$ and Ry receptors use a common functional calcium pool. The procedure employed was to deplete the ryanodine-sensitive calcium stores and test the ability of subsequently applied $InsP_3$ to mobilize further calcium from stores. It was established that depleting ryanodine-sensitive stores prevented $InsP_3$ from releasing further calcium. It is concluded that functionally, $InsP_3$ releases calcium from the same calcium pool utilized by Ry receptors in Purkinje cells.

## MATERIALS AND METHODS

### Preparation

Thin brain slices were prepared as previously described (Edwards et al., 1989). Briefly, 9–20-day-old rats were decapitated, and 300 $\mu$m sagittal cerebellar slices were made at the level of the vermis with a vibratome (Campden Instruments). The slices were incubated for 1 h at 34°C before use.

### Solutions

The composition of the extracellular solution was (mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, 11 glucose, and 1.5 $CaCl_2$. When bubbled with 95% $O_2$/5% $CO_2$, the solution had a pH of ~7.4. The composition of the intracellular solution was (mM): 145 K-gluconate, 8 KCl, 3 MgATP, 10 HEPES (K) (pH 7.2). The osmolality of the external solution was adjusted to 295 mOsmol $kg^{-1}$, and that of the internal solution was 287 mOsmol $kg^{-1}$. Fluo-3 (200 $\mu$M) (Molecular Probes, Eugene, OR), caged-$InsP_3$ (150 $\mu$M) (gift of Dr. Jeffery Walker), ryanodine (50 $\mu$M) (LC Laboratories, Woburn, MA), or ruthenium red (20 $\mu$M) was added to the internal solution when indicated. All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

### Electrical measurements

For recordings, a slice was transferred to the experimental chamber on the stage of a Zeiss Axioskop and was continuously perfused with the extracellular solution at room temperature (20–24°C). Slices were viewed with a Zeiss 63×, 0.9 NA infinity-corrected water immersion objective. The surfaces of visually identified Purkinje neurons were gently "cleaned" (Edwards et al., 1989), and tight-seal whole-cell patch-clamp recordings (Hamill et al., 1981) made with patch pipettes with an electrical resistance of 1.5–2.5 M$\Omega$. The series resistance upon achieving whole-cell configuration was between 4 and 6 M$\Omega$, and was compensated by at least 60% by the amplifier circuitry. Cells were voltage clamped at −70 mV and, with the solutions used, typically required less than −250 pA of holding current. Recording was terminated if the holding current became greater than −500 pA in any cell. For experiments we used a homemade voltage clamp amplifier and a homemade 16-bit D/A-A/D converter controlled with an IBM PC using custom-written software.

### Optical measurements

The $Ca^{2+}$ indicator Fluo-3, which was introduced into the cell via the whole-cell patch pipette, was excited with light from a tungsten halogen lamp directed through a 485-nm DF bandpass filter (full width half-maximum 22 nm) and routed through a 475-nm long pass 45° dichroic filter employing standard epifluorescence microscopy techniques. The emitted fluorescence, usually collected from the cell body and the dendritic tree, was guided through a 530-nm bandpass filter (full width half-maximum 30 nm) and focused on a photodiode detector (Hammamatsu) for quantitative measurements. The photodiode was connected to a homemade current-to-voltage converter, which had a response time constant of ~1 ms. The output of the photodiode current-to-voltage converter was protected by a pair of zener diodes to avoid saturation of the operational amplifier during the UV pulse. The signal from the photodiode was amplified, filtered at 2 kHz, passed through a pulse code modulator (PCM-701ES; Sony), and stored on videotape (SL-2700B; Sony) for further analysis. After achievement of whole-cell configuration, the content of the patch pipette was allowed to equilibrate with the cell before the start of the experimental procedures. The equilibration was assumed to be complete when the emitted Fluo-3 fluorescence had reached a plateau (usually within 5–15 min of going whole-cell). With the arrangements mentioned, electrical and optical recordings were made from cells for periods in excess of 90 min without detectable bleaching or damage from phototoxicity.

### Flash photolysis

A 238 Chadwick Helmuth Storbex (El Monte, CA) xenon arc lamp was used to produce UV pulses of ~1 ms in duration. The flash lamp power supply was modified to store about double the usual energy by increasing the bank of capacitors that stored the charge and increasing the number of diodes in the lamp housing. The capacitors could be charged to different voltages, allowing the discharge of variable intensities of UV light to photorelease known quantities of $InsP_3$ in the cytosol. With the aid of a 370-nm long pass 45° dichroic filter, the UV pulse shared the same light path as that employed by the fluorescence excitation light. The tungsten halogen coil and the UV flash arc were imaged on the back focal plane of the objective, constituting a Köhler illumination system, which illuminated the sample uniformly. All of the optical elements in the light path, aside from the objective and dichroic mirrors, were replaced with counterparts made of fused silica (Quantum Optics, Pom Fret, Connecticut) for enhanced UV transmission. The extent of photolysis was calibrated using the fluorescent pH indicator, 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF), taking advantage of the stoichiometric release of a proton with ATP during photolysis of caged MgATP (Walker et al., 1988), which has the same photolytic efficiency as caged $InsP_3$ (Walker et al., 1989). A round 250-$\mu$m-diameter quartz capillary was placed in the experimental chamber and viewed with the objective employed for experiments. The capillary was filled with a solution that contained 200 $\mu$M caged ATP, 20 $\mu$M BCECF, 1 mM dithiothreitol, and 3 mM $MgCl_2$, with the pH adjusted to 7.0. The fluorescence emitted by BCECF was measured before and after photolysis of caged ATP caused by a pulse of UV light of known intensity. Identical solutions to which known quantities of 1 M HCl were added were perfused through the capillary, the fluorescence emitted by the indicator was measured, and a calibration curve was constructed. The extent of photolysis was calculated by estimating the number of protons released by each pulse of UV light from the calibration curve. Up to ~65% photolysis of caged $InsP_3$ could be achieved with a single pulse of the flash lamp at full power. The flash lamp could be triggered either manually or by the computer controlling the voltage clamp amplifier.

## RESULTS

### Modification of Ry-Rs by ryanodine abolishes $InsP_3$ responses

Purkinje neurons were whole-cell voltage clamped with a patch pipette that contained an intracellular solution supplemented with the calcium indicator Fluo-3 and caged $InsP_3$. A short (1 ms) pulse of UV light was used to rapidly

photolyze (time constant ~3 ms; Walker et al., 1989) a known fraction of the caged $InsP_3$. The resulting changes in the Fluo-3 fluorescence and membrane conductance after photorelease of $InsP_3$ were measured. Fig. 1 shows changes in the Fluo-3 fluorescence after photorelease of $InsP_3$ in Purkinje neurons in the absence (Fig. 1 *A*) and presence (Fig. 1 *B*) of ryanodine. In the absence of ryanodine, as reported earlier (Khodakhah and Ogden, 1992, 1995), photorelease of $InsP_3$ resulted in the mobilization of $Ca^{2+}$ from intracellular stores reflected by an increase in the Fluo-3 fluorescence. The extent of calcium released depends on the $InsP_3$ concentration, and the Fluo-3 signal clearly indicates that more $Ca^{2+}$ was mobilized with the photorelease of 38 rather than 20 $\mu$M $InsP_3$. Photorelease of yet a higher concentration of $InsP_3$ (57 $\mu$M) resulted in a Fluo-3 signal that remained elevated for a longer period, although the absolute changes in the $Ca^{2+}$ concentration cannot be obtained with accuracy, because of saturation of the indicator. Although quantitative descriptions of the changes in the intracellular $Ca^{2+}$ after photorelease of $InsP_3$ requires the use of a lower affinity indicator such as furaptra (Khodakhah and Ogden, 1992, 1995), a good indication of the large magnitude of the $Ca^{2+}$ release can be obtained by comparing the $InsP_3$-mediated changes in the Fluo-3 signal with the changes brought about by a train of depolarizations. Fig. 1 *C* shows the changes in the Fluo-3 fluorescence when a train of 14 100-ms depolarizations to 0 mV was applied to the cell at 5 Hz. The influx of $Ca^{2+}$ through voltage-gated calcium channels increases the intracellular concentration of $Ca^{2+}$. The whole-cell current records indicated that each depolarization resulted in the activation of approximately similar numbers of voltage-activated calcium channels (data not shown). Whereas the first few depolarizations result in the same increase in the Fluo-3 fluorescence because the intracellular $Ca^{2+}$ remains within the linear fluorescence range of the indicator, subsequent voltage steps result in smaller changes as Fluo-3 saturates. Comparison of Fig. 1 *A* and Fig. 1 *C* demonstrates the large magnitude of the $InsP_3$-evoked $Ca^{2+}$ release, and indicates that the indicator was saturated during the $InsP_3$-evoked calcium responses.

The effect of ryanodine on $InsP_3$ responses was studied by including 50 $\mu$M ryanodine in the patch pipette and allowing it to equilibrate with the cell. Ryanodine modifies Ry-Rs by clamping its channel open at a subconductance state (Smith et al., 1988). Application of ryanodine has been demonstrated to abolish caffeine responses (Kano et al., 1995), and calcium-induced calcium release (CICR) (Llano et al., 1994), without significantly changing the free intracellular calcium concentration in Purkinje neurons (Llano et al., 1994; Kano et al., 1995). In the presence of ryanodine, it is expected that the increased $Ca^{2+}$ efflux through the modified Ry-Rs would substantially reduce the $Ca^{2+}$ concentration in the ryanodine-sensitive stores. If $InsP_3$ receptors use a $Ca^{2+}$ pool separate from that used by Ry-Rs, $InsP_3$-evoked calcium responses should be unaffected by the presence of ryanodine. Alternatively, if $InsP_3$ and Ry receptors use the same $Ca^{2+}$ pool, the $InsP_3$-evoked calcium responses should be substantially smaller in the presence of ryanodine. Fig. 1 *B* shows the photorelease of 20, 38, and 57 $\mu$M $InsP_3$ in a Purkinje neuron voltage clamped with a patch pipette containing 50 $\mu$M ryanodine. As demonstrated by the small changes in the Fluo-3 fluorescence, none of the

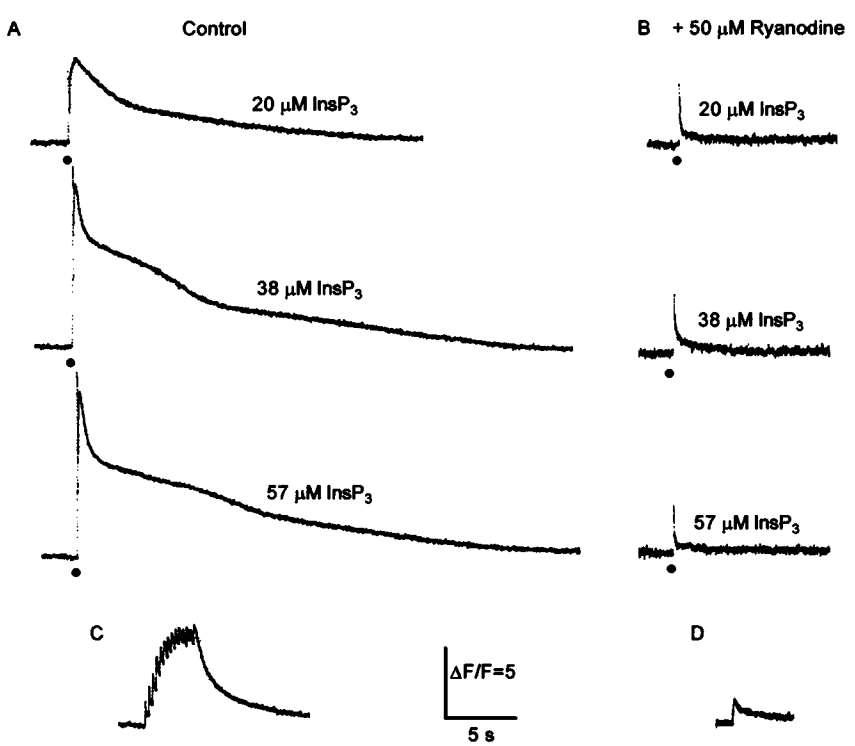


FIGURE 1 Ryanodine abolishes $InsP_3$-evoked calcium mobilization in Purkinje neurons. (*A*) Changes in the Fluo-3 fluorescence emitted from a voltage-clamped Purkinje neuron after photorelease of $InsP_3$ or a train of depolarizing voltage pulses. One-millisecond pulses of UV light of varying intensity were delivered (where indicated by the *filled circles*) to rapidly photorelease 20 $\mu$M, 38 $\mu$M, and 57 $\mu$M $InsP_3$ in the cytosol of a voltage-clamped Purkinje neuron. $InsP_3$-evoked $Ca^{2+}$ release from stores is clearly evident from the rise in the Fluo-3 signal after an initial fast optical artifact arising from the UV pulse. (*B*) Experiment similar to the one above, but in the presence of 50 $\mu$M ryanodine. Photorelease of 20 $\mu$M, 38 $\mu$M, and 57 $\mu$M $InsP_3$ failed to produce a significant change in the Fluo-3 signal. (*C*) Changes in the Fluo-3 signal due to a train of 14 100-ms-long depolarizing pulses to 0 mV in the cell shown in *A*. Whereas the first few pulses result in comparable increases in the emitted fluorescence, subsequent changes are smaller as $[Ca^{2+}]_i$ increases beyond the $K_D$ of the indicator. (*D*) The response of the indicator to a single 100-ms depolarizing pulse to 0 mV in the same cell as in *B*. After depletion of ryanodine-sensitive $Ca^{2+}$ stores, $InsP_3$ fails to mobilize further calcium from the stores (AU106A cells 1 and 2).

$InsP_3$ pulses resulted in a substantial release of $Ca^{2+}$ from the stores, although the indicator could reliably detect changes in intracellular free $Ca^{2+}$ after a single 100-ms depolarization of the cell to 0 mV (Fig. 1 *B*). Cells voltage clamped in the presence of ryanodine did not require a significantly different holding current and demonstrated normal sodium, potassium, and calcium currents. The inability of $InsP_3$ to mobilize calcium is, therefore, a consequence of the depletion of the ryanodine sensitive stores and not the result of the poor health of the cell or saturation of the indicator.

## Ryanodine modifies activated Ry channels

It is well established that ryanodine only modifies open Ry-Rs. During the time taken for the indicator to equilibrate with the cell after going whole-cell (5–15 min), random openings of the Ry-Rs would allow for modification of a fraction of these receptors. The concentration of $Ca^{2+}$ in the stores would be determined by the balance between the influx rate of $Ca^{2+}$ into the stores (presumably mainly by the calcium pumps), and the rate of efflux, which in the presence of ryanodine is probably dominated by the exodus of $Ca^{2+}$ through the modified Ry-Rs. In the majority of cells (five of seven), by the time the concentration of indicator had reached steady state (judged by the leveling off of the emitted fluorescence), photorelease of $InsP_3$ failed to significantly increase cytosolic free $Ca^{2+}$ concentration (e.g., Figs. 1 *B* and 3 *B*). An interpretation of this finding is that the fraction of ryanodine receptors modified during the loading was large enough to substantially reduce the concentration of $Ca^{2+}$ in the stores. In two of the seven cells, however, $InsP_3$ pulses resulted in small changes in the Fluo-3 fluorescence when the indicator had equilibrated with the cell. Fig. 2 *A* shows the changes in the fluorescence with three consecutive pulses of $InsP_3$ in one of these cells. These responses were much smaller than those seen in the absence of ryanodine (compare with the depolarizing trains in Fig. 2 *B* and the same in Fig. 1), indicating that the stores had, at least partially, lost their calcium content. It was postulated that the concentration of $Ca^{2+}$ in the stores could be made even lower if more of the Ry-Rs were to be modified, thus increasing the rate of $Ca^{2+}$ efflux. Because ryanodine modifies open channels, more of the Ry-Rs would be modified if they were forced to open by elevating intracellular $Ca^{2+}$. One way to increase intracellular $Ca^{2+}$ is by applying a train of depolarizing pulses to allow $Ca^{2+}$ entry through the voltage-activated $Ca^{2+}$ channels. This hypothesis was examined as shown in Fig. 2, *B* and *C*; the intracellular $Ca^{2+}$ was elevated by the application of two depolarizing trains and the ability of $InsP_3$ to mobilize $Ca^{2+}$ tested in the same two cells. In both cells, $InsP_3$ failed to mobilize $Ca^{2+}$ from the intracellular stores after the depolarizing trains. It seems plausible, therefore, to conclude that modification of more ryanodine receptors resulted in a greater reduction in the $Ca^{2+}$ concentration of the stores.

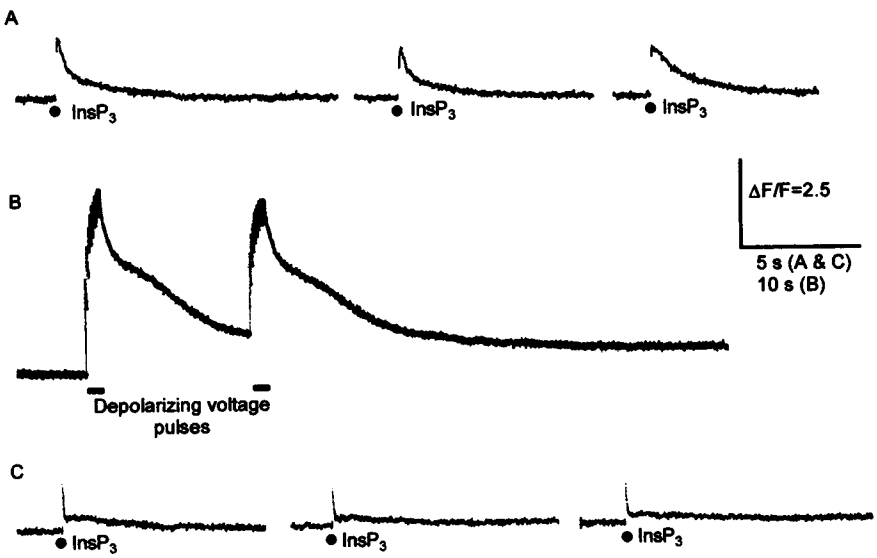


FIGURE 2 Ryanodine modifies open ryanodine channels: changes in the $[Ca^{2+}]_i$ in a Purkinje neuron voltage clamped in the presence of 50 $\mu$M ryanodine. In this and a second cell, small responses to the photorelease of $InsP_3$ were detected when the contents of the patch pipette had equilibrated with the cell (judged from the Fluo-3 signal). (*A*) Three pulses of UV light photoreleased 38 $\mu$M $InsP_3$ (where indicated by the *filled circles*). $InsP_3$ evoked small changes in the $[Ca^{2+}]_i$, as demonstrated by the increase in the Fluo-3 signal. These responses were much smaller than that in the absence of ryanodine, indicating that the stores had lost part of their $Ca^{2+}$ content. (*B*) Two trains of depolarizing voltage pulses were applied to increase $[Ca^{2+}]_i$ by opening voltage-activated calcium channels in the plasma lemma. It was postulated that high $[Ca^{2+}]_i$ would activate the remaining ryanodine receptors, allowing their modification by ryanodine. After such treatment, the increased rate of calcium efflux from the stores due to the modified ryanodine receptors would reduce the concentration of $Ca^{2+}$ in the stores even more. The $[Ca^{2+}]_i$ returned to baseline ~60 s after the end of the trace shown. (*C*) Photorelease of 38 $\mu$M $InsP_3$, after return of $[Ca^{2+}]_i$ to its resting level, failed to mobilize more calcium from the stores. Apparently, modification of more Ry-Rs by ryanodine during the depolarizing trains resulted in depletion of $Ca^{2+}$ stores (AU126A cell 3).

## Ruthenium red does not significantly alter $InsP_3$ responses

One possibility that needs to be ruled out is whether CICR contributes significantly to the $InsP_3$-evoked calcium responses. A process may be imagined whereby after photorelease of $InsP_3$, a small efflux of $Ca^{2+}$ from $InsP_3$ receptors activates many ryanodine receptors. It can then be reasoned that the bulk of $Ca^{2+}$ released from the stores after photorelease of $InsP_3$ is through the Ry-Rs and not the $InsP_3$-Rs. Given such a scenario, disruption of CICR with ryanodine may result in smaller, or diminished, $InsP_3$-evoked calcium responses. Alternatively, it may be imagined that $Ca^{2+}$ release from Ry-Rs is necessary to "boost" the $InsP_3$-evoked calcium release by increasing the $InsP_3$ receptor open probability (Iino, 1990; Bezprozvanny et al., 1991; Finch et al., 1991). To rule out these possibilities, ruthenium red, a Ry-R antagonist (Lai et al., 1988; Smith et al., 1988; Ehrlich and Watras, 1988; Bezprozvanny et al., 1991), was added to the intracellular patch pipette. Both calcium-induced calcium release from ryanodine receptors (Llano et al., 1994) and calcium increase after application of caffeine have been shown to be abolished by ruthenium red in Purkinje cells. Under these conditions, aside from a slight reduction, $InsP_3$-evoked calcium responses were not significantly affected (Fig. 3 *A*). An additional indication of the extent of calcium release by $InsP_3$ is the $Ca^{2+}$-activated potassium conductance present in Purkinje neurons. Whereas inclusion of ryanodine in the patch pipette abolishes changes in the Fluo-3 fluorescence and membrane conductance after photorelease of $InsP_3$ (Fig. 3 *A*), a large outward current was recorded when $InsP_3$ was photoreleased in the presence of ruthenium red (Fig. 3 *B*), indicating that Ry-Rs do not play a major role in $InsP_3$-evoked calcium release in Purkinje neurons.

Although it is clear that CICR does not contribute significantly to $InsP_3$-evoked calcium release, it is important to recognize that Fluo-3 is not a suitable indicator for quantitative analysis of large changes in $Ca^{2+}$ concentration. The exact contribution of CICR to $InsP_3$ responses, therefore, must be evaluated with a lower affinity indicator such as Furaptra. Nevertheless, for the purposes of this report, it can be conservatively concluded that the effects of ryanodine cannot be explained with a CICR hypothesis and that ryanodine abolishes $InsP_3$-evoked $Ca^{2+}$ release by depleting the calcium pools.

## Partial refilling of the stores

As reasoned earlier, in the presence of ryanodine the rate of calcium efflux from the stores is increased, thus lowering the calcium concentration in them. We considered the possibility that if the cytosolic calcium concentration were to be elevated momentarily, one would increase the amount of calcium pumped into the stores and perhaps partially refill them. Consequently, it should be possible to demonstrate $InsP_3$-evoked calcium release after such partial refilling. This hypothesis was tested on three cells, which all gave qualitatively similar results.

Fig. 4 shows one such experiment, where 50 $\mu$M ryanodine was included in the patch pipette. The absence of a significant change in the Fluo-3 signal after photorelease of 75 $\mu$M $InsP_3$ is shown in Fig. 4 *A*. A 4-s train of 10 100-ms depolarizing pulses to 0 mV was applied to the cell (Fig. 4 *B*) to increase the intracellular free calcium concentration. When $Ca^{2+}$ returned to resting levels (judged by the Fluo-3 signal), 38 $\mu$M $InsP_3$ was photoreleased (Fig. 4 *C*). This application of $InsP_3$ resulted in a small but detectable calcium release from the stores, suggesting that the stores were

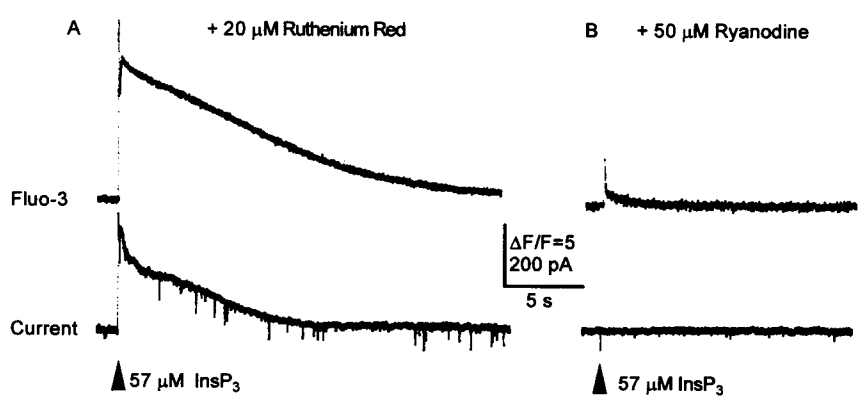


FIGURE 3 CICR does not play a major role during $InsP_3$-evoked calcium release. (*A*) 20 $\mu$M ruthenium red, a ryanodine receptor antagonist, was added to the intracellular patch pipette solution of a voltage-clamped Purkinje neuron. Photolytic release of 57 $\mu$M $InsP_3$ (indicated by the *arrowhead*) caused a large increase in the Fluo-3 fluorescence as $[Ca^{2+}]_i$ was elevated by $Ca^{2+}$ release from the intracellular stores (*top records*). Elevation of the $[Ca^{2+}]_i$ activates calcium-activated potassium channels, which resulted in an outward current (*bottom records*). It is clear that blocking Ry-Rs with ruthenium red did not have a significant effect on $InsP_3$-evoked calcium release from stores. (*B*) Changes in the Fluo-3 fluorescence and membrane current in a Purkinje neuron voltage clamped in the presence of 50 $\mu$M ryanodine. Photolytic release of 57 $\mu$M $InsP_3$ did not result in a significant change in the Fluo-3 fluorescence or alter the membrane conductance. Ryanodine abolishes $InsP_3$-evoked $Ca^{2+}$ release, not by preventing CICR, but by depleting the stores (AU236A cells 1 and 2).

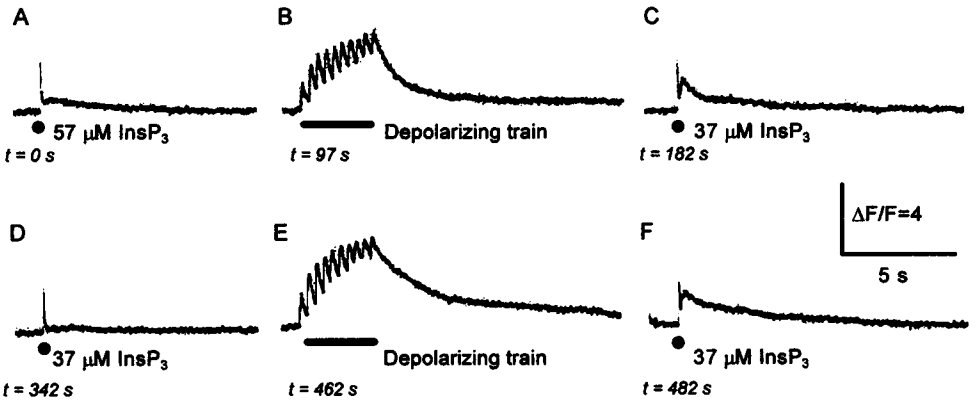


FIGURE 4 Partial refilling of the stores by elevation of $[Ca^{2+}]_i$ in the presence of ryanodine. (*A*) Photorelease of 76 μM $InsP_3$ (indicated by the *filled circle*) resulted in little change in the $[Ca^{2+}]_i$ in the presence of 50 μM ryanodine. (*B*) A train of 10 100-ms depolarizing pulses to 0 mV (*solid bar*) was applied to elevate $[Ca^{2+}]_i$. (*C*) Immediately after the return of $[Ca^{2+}]_i$ to its resting level, 38 μM $InsP_3$ was photoreleased, causing a small increase in $[Ca^{2+}]_i$. Clearly the stores had partially refilled with $Ca^{2+}$ during the train of depolarizing pulses. (*D*) A subsequent increase in $InsP_3$ (38 μM) 3 min later failed to mobilize $Ca^{2+}$ in the same cell. (*E*) Repeat of the depolarizing pulses a second time to partially refill the stores restored the ability of $InsP_3$ in mobilizing calcium (*F*) (AU126A cell 1).

partially refilled during the depolarizing pulses. Fig. 4 *D* shows the effect of photorelease of 38 μM $InsP_3$ ~3 min later. $InsP_3$ failed to produce a significant change in the Fluo-3 signal, presumably because during this delay the stores had again emptied because of the continuous exodus of calcium through the modified ryanodine receptors. Repeat of the depolarizing pulses and a fourth application of $InsP_3$ (Fig. 4, *E* and *F*) demonstrated the possibility of a second partial refilling of the stores. It needs to be mentioned that such rapid successive photorelease of $InsP_3$ does not allow sufficient time for reequilibration of caged $InsP_3$ present in the patch pipette and the cytosol. The concentration of $InsP_3$ released after the first flash, therefore, would have been less than that indicated, strengthening the case for partial refilling of the stores.

## DISCUSSION

The aim of this study was to establish whether $InsP_3$ and ryanodine receptors use two functionally distinct intracellular $Ca^{2+}$ pools in Purkinje neurons. Our main finding is that depletion of the ryanodine-sensitive calcium pools renders $InsP_3$ incapable of releasing more calcium from the stores. The simplest interpretation of our results is that $InsP_3$ receptors use the same functional $Ca^{2+}$ pool as that employed by the ryanodine receptors.

Whereas inositol trisphosphate and ryanodine receptors are found in cerebellar Purkinje neurons (Ellisman et al., 1990; Walton et al., 1991; Sharp et al., 1993), based on light and electron microscopic studies, it is evident that neither is distributed uniformly in the cell (Ellisman et al., 1990; Walton et al., 1991; Takei et al., 1992; Sharp et al., 1993). Both receptors are found in the entire cell, with the exception of the dendritic spines, where $InsP_3$, but not Ry receptors, are present (Ellisman et al., 1990; Walton et al., 1991; Takei et al., 1992; Sharp et al., 1993). The heterogeneous distribution of $InsP_3$ and Ry receptors in Purkinje neurons has suggested to some the existence of functionally separate $Ca^{2+}$ stores, preferentially activated by different second messengers (Ellisman et al., 1990; Walton et al., 1991; Takei et al., 1992; Sharp et al., 1993; Nori et al., 1993). In support of this hypothesis, subfractionation of cerebellar microsomes has shown that vesicles bearing the $InsP_3$-Rs are separated from those containing the Ry-Rs (Volpe et al., 1991; Nori et al., 1993). To date, however, the functional heterogeneity of $InsP_3$ and ryanodine-sensitive calcium stores has not been directly demonstrated in Purkinje neurons. Experiments reported here attempted to address this question by depleting ryanodine-sensitive stores and testing the ability of subsequently photoreleased $InsP_3$ to mobilize $Ca^{2+}$. To deplete ryanodine-sensitive stores, we took advantage of the fact that ryanodine modifies its receptor channel by clamping it in an open subconductance state (Smith et al., 1988). It has been demonstrated that ryanodine abolishes calcium-induced calcium release (Llano et al., 1994) and responses to the application of caffeine (Kano et al., 1995) in Purkinje neurons. The effect of ryanodine is presumably due to the continuous exodus of $Ca^{2+}$ through the modified Ry-Rs, which results in a considerable reduction in the concentration of $Ca^{2+}$ in the stores. Interestingly, we discovered that the application of ryanodine significantly reduced $InsP_3$-evoked elevation of intracellular calcium.

Ryanodine did not have any adverse effects on the health of the Purkinje neurons; it did not notably alter the resting intracellular free calcium concentration, nor did it alter the voltage-activated ionic currents. In addition to the present work, several other studies have also demonstrated that the addition of ryanodine does not elevate $[Ca^{2+}]_i$ in Purkinje neurons (Llano et al., 1991a; Kano et al., 1995). The inability of $InsP_3$ to mobilize $Ca^{2+}$ in the presence of ryanodine, therefore, was not due to the inhibition of $InsP_3$ receptors by elevated $[Ca^{2+}]_i$ (Bezprozvanny et al., 1991; Khodakhah

and Ogden, 1995). This notion is further supported by experiments where $InsP_3$-evoked calcium responses were obtained in the presence of ryanodine after partial refilling of the stores (see Fig. 4). In these experiments, stores were partially refilled with a train of depolarizing voltage pulses, which elevated $[Ca^{2+}]_i$ for a few seconds. The demonstration of $InsP_3$-evoked calcium release after this procedure argues against a nonspecific effect by ryanodine, or the possibility that ryanodine inhibited $InsP_3$-evoked calcium release by increasing $[Ca^{2+}]_i$. In one experiment we coapplied 20 $\mu$M ruthenium red together with 50 $\mu$M ryanodine. In this cell, photolytic release of $InsP_3$ resulted in prominent calcium release from the intracellular stores, presumably because the ryanodine-modified ryanodine receptors were blocked with ruthenium red and thus failed to deplete the store.

To understand the mechanism of the effect of ryanodine on $InsP_3$-evoked calcium release in more detail, we considered the possibility that ryanodine receptors themselves may play a crucial part during $InsP_3$-evoked calcium responses in Purkinje neurons. This could occur if a large portion of the calcium released from the stores after photorelease of $InsP_3$ stemmed from the activation of many ryanodine receptors, mediated by $Ca^{2+}$ released through $InsP_3$ receptors. Alternatively, a scheme may be imagined whereby $Ca^{2+}$ release from Ry-Rs is necessary to "boost" $InsP_3$-evoked calcium release by increasing the $InsP_3$ receptor open probability. It is well documented that calcium modulates $InsP_3$ channels, having a positive feedback on their open probability at low concentrations (Iino, 1990; Bezprozvanny et al., 1991; Finch et al., 1991). To rule out these possibilities, experiments were carried out in which the ryanodine receptor antagonist ruthenium red (Lai et al., 1988; Smith et al., 1988; Ehrlich and Watras, 1988; Bezprozvanny et al., 1991) was used to block ryanodine receptors. This procedure failed to abolish $InsP_3$-evoked $Ca^{2+}$ release or significantly reduce the calcium-activated K conductance, and provided evidence against a major contribution by Ry-Rs in $InsP_3$-evoked calcium responses in Purkinje neurons.

The simplest interpretation of the data presented is that depletion of the ryanodine-sensitive calcium pool also depletes the $InsP_3$-sensitive calcium pool. The direct implication of this finding is that, in Purkinje neurons, all stores that express functional $InsP_3$ receptors also contain functional ryanodine receptors (Fig. 5, *D* and *E*). This conclusion is supported by the fact that in Purkinje neurons, $InsP_3$ and ryanodine receptors have overlapping distributions (Walton et al., 1991; Sharp et al., 1993), and often, $InsP_3$ and ryanodine receptors are found to coexist on the same membrane (Walton et al., 1991). Our results argue against the presence of separate $InsP_3$ and ryanodine calcium pools, either as physically isolated calcium stores or as subcompartments in the lumen of a continuous store in the soma and dendritic shafts (Fig. 5, *A–C*, but see next paragraph). We are unable, however, to rule out the possibility of coexistence of separate stores containing only ryanodine receptors (Fig. 5 *E*). To test such a possibility, one would need to deplete the $InsP_3$-sensitive stores and test whether it is possible to release further calcium from stores by activating ryanodine receptors. We are not aware of any procedures that would allow one to deplete $InsP_3$-sensitive stores completely and still permit study of ryanodine channels in intact Purkinje neurons. It is clear from the data presented, nevertheless, that all stores that express functional $InsP_3$-gated calcium release channels also express functional ryanodine channels.

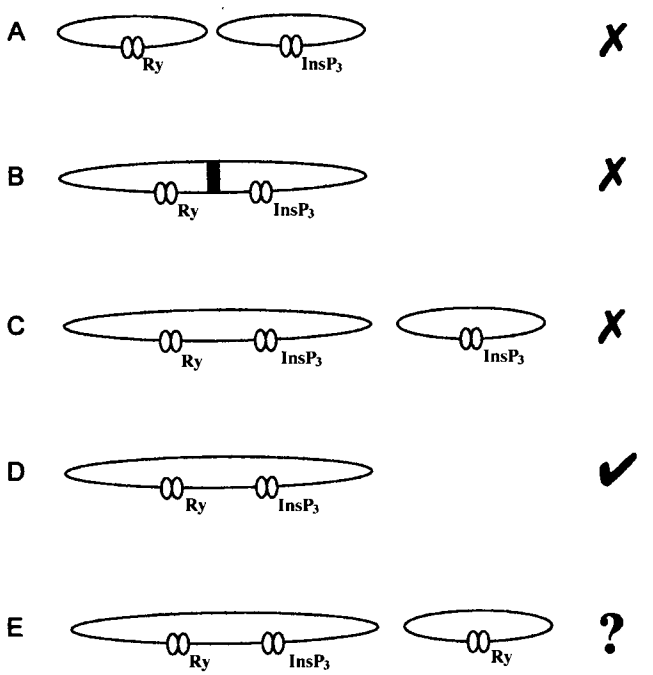


FIGURE 5 Calcium stores in a cerebellar Purkinje neuron. The cartoon portrays several possible configurations of the $Ca^{2+}$ stores in a Purkinje neuron. Experiments outlined in this report are not compatible with the existence of functionally separate, $InsP_3$- and ryanodine-sensitive $Ca^{2+}$ pools, either as physically separate stores (*A*) or as lumenal subcompartments in a continuous store (*B*). Interpretation of our experiments necessitates the presence of functional Ry-Rs on all $Ca^{2+}$ stores where $InsP_3$-R is present (*D*), rejecting the possibility of existence of separate $InsP_3$ stores (*C*). The data presented, however, do not rule out the coexistence of $Ca^{2+}$ pools expressing only ryanodine receptors (*E*).

Additional consideration should be given to the properties of calcium stores in dendritic spines. It is well documented that dendritic spines in Purkinje neurons possess $InsP_3$ receptors, yet are devoid of Ry-Rs. It is conceivable that the stores present in the spines are isolated, and that $InsP_3$-evoked calcium responses in the spines are not affected by the depletion of ryanodine-sensitive stores. Given such assumption, one may imagine that we failed to record these responses in the presence of ryanodine because of the limited signal originating from the small volume of the spines. To address this point rigorously, similar experiments need to be performed employing the higher resolution of confocal or two-photon excitation microscopy, but we believe this to be an unlikely scenario for the following reasons. To maximize our ability to detect small changes, we used a relatively bright calcium indicator, Fluo-3, which

has high affinity for calcium ions (about 450 nM *in vitro*). $InsP_3$-evoked changes in $[Ca^{2+}]_i$ are quite substantial, often reaching tens of micromoles/liter (Khodakhah and Ogden, 1992, 1995), and result in large changes in the Fluo-3 fluorescence. Inclusion of ryanodine in the intracellular solution, however, effectively abolished $InsP_3$-evoked changes in Fluo-3 fluorescence, which suggests that all stores expressing $InsP_3$ receptors were depleted. In addition, the membrane of the endoplasmic reticulum in the dendritic spines has been shown to be continuous with that in the dendritic shaft where Ry-Rs are present (Walton et al., 1991). For $InsP_3$-evoked responses to be unaffected by the depletion of ryanodine-sensitive stores, one would need to imagine that the lumen of these stores is compartmentalized; no physical evidence for such partitions has been documented.

In summary, we provide evidence that in Purkinje neurons $InsP_3$ and ryanodine receptors use a common functional calcium pool. It would be of interest to examine whether functionally distinct $Ca^{2+}$ pools exist in other neurons, for example, in cortical pyramidal neurons, where $InsP_3$ and Ry-Rs are clearly localized to different parts of the cell. Our finding that $InsP_3$ and ryanodine receptors use a common functional $Ca^{2+}$ pool does not make the possibility of localized $Ca^{2+}$ release in response to different second messengers less likely. Such a process, one may speculate, could be regulated by several different strategies. For example, $InsP_3$ and ryanodine receptors could simply be concentrated at different locations. Furthermore, calcium release from the stores may be spatially localized because of the limited range and slow diffusion rate of locally produced, spatially confined second messengers and $Ca^{2+}$ ions.

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