Impaired Calcium Release in Cerebellar Purkinje Neurons Maintained in Culture

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abstract Cerebellar Purkinje neurons demonstrate a form of synaptic plasticity that, in acutely prepared brain slices, has been shown to require calcium release from the intracellular calcium stores through inositol trisphosphate ($InsP_3$) receptors. Similar studies performed in cultured Purkinje cells, however, find little evidence for the involvement of $InsP_3$ receptors. To address this discrepancy, the properties of $InsP_3$- and caffeine-evoked calcium release in cultured Purkinje cells were directly examined. Photorelease of $InsP_3$ (up to 100 μM) from its photolabile caged analogue produced no change in calcium levels in 70% of cultured Purkinje cells. In the few cells where a calcium increase was detected, the response was very small and slow to peak. In contrast, the same concentration of $InsP_3$ resulted in large and rapidly rising calcium responses in all acutely dissociated Purkinje cells tested. Similar to $InsP_3$, caffeine also had little effect on calcium levels in cultured Purkinje cells, yet evoked large calcium transients in all acutely dissociated Purkinje cells tested. The results demonstrate that calcium release from intracellular calcium stores is severely impaired in Purkinje cells when they are maintained in culture. Our findings suggest that cultured Purkinje cells are an unfaithful experimental model for the study of the role of calcium release in the induction of cerebellar long term depression.

key words: inositol trisphosphate • ryanodine • long term depression • synaptic plasticity • calcium stores

INTRODUCTION

Repeated concurrent activation of the two major excitatory inputs to cerebellar Purkinje cells, the climbing fibers and the parallel fibers (PFs),[1] results in the long-term depression (LTD) of the PF synaptic response (Ito, 1984), a phenomenon that is thought to impart to the cerebellum its ability to learn motor tasks. There is good evidence that inositol trisphosphate ($InsP_3$)–evoked calcium release plays a role in the induction of LTD. Activation of the type 1 metabotropic glutamate receptors, which are coupled to phosphoinositide turnover, is necessary for the induction of LTD (Conquet et al., 1994). Purkinje cells have the highest density of $InsP_3$ receptors in the central nervous system (Worley et al., 1989), these being predominantly type 1 (De Smedt et al., 1994). $InsP_3$ receptors are present in the soma, dendrites, and dendritic spines of Purkinje cells (Ellisman et al., 1990). In cerebellar slices, photorelease of $InsP_3$ can substitute for the activation of PFs in the induction of LTD (Khodakhah and Armstrong, 1997a; Finch and Augustine, 1998). The $InsP_3$ receptor antagonist heparin (Khodakhah and Armstrong, 1997a) and a specific type 1 $InsP_3$ receptor antibody (Inoue et al., 1998) block the induction of LTD. Mice with a disrupted type 1 $InsP_3$ receptor gene completely lack LTD (Inoue et al., 1998).

Cultures of dissociated neurons are frequently used as simplified systems with which to study the cellular basis of neuronal plasticity. Long term depression of currents produced by ionophoretically applied glutamate has been described in cultured Purkinje cells (culture-LTD) (Linden et al., 1991). This form of plasticity has been assumed to share the same properties as LTD of PF synaptic responses in cerebellar slices, and cultured Purkinje cells are routinely used to identify second messenger pathways involved in LTD. However, recent results obtained using cultured Purkinje cells are at odds with those obtained from cerebellar slices: in cultured Purkinje cells, a selective and potent $InsP_3$ receptor antagonist, xestospongin c, does not affect the induction of long term depression (Narasimhan et al., 1998). Thus, the $InsP_3$ signaling pathway does not seem to be necessary for the induction of culture-LTD, yet is essential in cerebellar slices for the induction of long-term depression of PF synaptic inputs. One explanation for this discrepancy is that, in culture, release of calcium from intracellular stores is reduced while other second messenger signaling pathways associated with LTD are upregulated as compensation. Here we directly examine the properties of $InsP_3$- and caffeine-evoked calcium responses in both cultured and acutely dissociated

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[1]*Abbreviations used in this paper:* $InsP_3$, inositol trisphosphate; LTD, long-term depression; PF, parallel fiber.

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Purkinje cells. We find that intracellular calcium release via both $InsP_3$ and ryanodine receptors is severely impaired when Purkinje cells are maintained in culture. Our results indicate that the properties of at least one of the intracellular signaling systems thought to be important in LTD is greatly altered in cultured Purkinje cells and that cultured cells may be an unfaithful experimental model for the cerebellar plasticity seen in vivo.

MATERIALS AND METHODS

Cell Culture

Cerebelli were removed from CD1 mice anaesthetized with Nembutal (50 mg/kg, i.p.) at embryonic days 16–18, dissociated by the method of Schilling et al. (1991), and plated on glass coverslips coated with polyethyleneimine. Cells were initially plated in culture medium (Basal Medium Eagle supplemented as described; Schilling et al., 1991) containing 5% horse serum. After 24–36 h cultures were switched to serum free medium. Cultures were then fed with serum-free medium every 4 d. All tissue culture reagents were obtained from GIBCO BRL, with the exception of aprotinin and bovine serum albumin (Sigma Chemical Co.). Experiments were done on 10 different culture dishes from three separate culture preparations.

Identification of Purkinje Neurons in Culture

To identify Purkinje neurons in culture, we labeled a few cultures with a monoclonal antibody to calbindin (Sigma Chemical Co.). At 7 d in vitro, >30% of neurons were positive for calbindin. The cell bodies of the calbindin-positive neurons were larger than (>20 μm) the calbindin-negative neurons, and the cells had more than two primary dendrites. Under bright-field illumination used for electrophysiological studies, Purkinje neurons were identified by their large, high profile cell bodies and the presence of more than two primary dendrites. Our visual identification of large-size neurons was confirmed electrophysiologically from their membrane capacitance. The average membrane capacitance of the cultured Purkinje cells was 13.2 ± 1.4 pF (SEM, $n = 10$), and the average cell input resistance was 691 ± 207 MΩ (SEM, $n = 10$). All cells included in this study exhibited large (>1.5 nA) rapidly activating inward currents upon depolarization to −40 mV.

Dissociated Purkinje Cells

Dissociated cells were prepared with the protocol developed by Mintz and Bean (1993). CD1 mice at postnatal days 10–16 were anaesthetized with Metafane by inhalation, and then decapitated. Cerebelli were removed, minced, and incubated in 10 ml dissociation solution (mM: 82 Na_2SO_4, 30 K_2SO_4, 5 $MgCl_2$, 10 HEPES, 10 glucose) containing 3 mg/ml protease III (Sigma Chemical Co.) at 37°C for 5–10 min, depending on the age of the mice. Tissue was removed from the enzyme solution and washed once with 5 ml dissociation solution containing bovine serum albumin (Sigma Chemical Co.) and trypsin inhibitor (GIBCO BRL), and then maintained in dissociation solution at room temperature. Just before recording, cells were dissociated by trituration through a fire-polished Pasteur pipette and allowed to stick to a glass coverslip mounted in the recording chamber. Purkinje neurons were unambiguously identified by their large size, and the presence of a single large proximal dendrite. Experiments were done on 10 neurons, each from a different animal. All experimental protocols used in this study where approved by the University of Colorado Health Sciences Center Animal Care and Use Committee.

Whole-Cell Voltage-Clamp Recordings

The composition of the extracellular solution was (mM): 140 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4. The intracellular solution contained 125 Kgluconate, 20 KCl, 10 KHEPES, and 3 MgATP, pH 7.2. The internal solution also contained 200 μM Fluo-3 (Molecular Probes) and 150 μM caged inositol trisphosphate (Walker et al., 1989). The caged $InsP_3$ was synthesized in the laboratory. The osmolality of the extracellular solution ranged from 295 to 300 mOsmol · kg^{-1} while the internal solution was 290 mOsmol · kg^{-1}. The preparation was continuously superperfused with external solution at room temperature. Neurons were visualized on a modified Axiovert 25, using a 1.35 N.A., 40× oil immersion objective (Carl Zeiss, Inc.,). Whole-cell patch-clamp recordings (Hamill et al., 1981) were made with pipettes with a resistance of 1.8–3.0 MΩ. The voltage error due to series resistance at the peak of responses was always <10 mV. Cells were voltage clamped at −80 mV with a homemade voltage-clamp amplifier. Data were recorded with an A/D, D/A converter (PCI-MIO-16XE-10; National Instruments) and an IBM computer using custom-written software. Reagents were obtained from Fisher Chemical unless otherwise indicated. Data from 22 neurons were analyzed for this study.

Optical Measurements

Fluorescence measurements were made using the Ca^{2+} indicator Fluo-3, which was introduced into the cells via the whole-cell patch pipette. Light from a tungsten halogen lamp passed through a monochromator (Cairn Instruments), which restricted the excitation wavelength to 485 ± 20 nm. The excitation light was transmitted to the epifluorescence port of the microscope via a liquid light guide. The end of the light guide was focused on the specimen plane. Emitted light was collected through a 530 ± 15-nm bandpass filter and quantitatively measured with a photon counting photomultiplier (Electron Tubes) using the PCI-MIO-16XE-10 counter board and custom-written software. A pinhole in the emitted light path limited the size of the field sampled by the photomultiplier to an area just slightly larger than that of the soma of the cell.

Flash Photolysis

A xenon arc lamp (Cairn Research) was used to produce UV pulses of ~1 ms in duration. The energy stored in the flash lamp power supply could be adjusted to vary the intensity of light and the amount of $InsP_3$ uncaged. UV light was transmitted to the microscope via a 3-mm-diameter liquid light guide, and with the aid of a dichroic mirror shared the same light path as that employed by the fluorescence excitation light. A liquid crystal shutter (Display Tech, Inc.), positioned in front of the photomultiplier was activated for 8 ms during the flash to prevent saturation of the photomultiplier. The extent of photolysis was calibrated using a fluorescent pH indicator taking advantage of the stoichiometric release of a proton with ATP during photolysis of caged MgATP, which has the same photolytic efficiency as caged $InsP_3$ (Walker et al., 1989). Caged $InsP_3$, or the photolytic by-products of caged $InsP_3$ do not release calcium or interfere with calcium release at concentrations up to 100 μM in hepatocytes or rat Purkinje cells (Khodakhah and Ogden, 1995).

RESULTS

We characterized the properties of $InsP_3$-evoked calcium transients in Purkinje cells in primary cultures prepared and maintained with the same protocol as for

LTD experiments (we thank Dr. David Linden, Johns Hopkins University School of Medicine, Baltimore, MD) for providing the detailed culture protocol). Purkinje cells maintained 10–16 d in culture were whole-cell voltage clamped with patch pipettes containing both caged $InsP_3$ and the fluorescent calcium indicator Fluo-3. The contents of the patch pipette solution were allowed to equilibrate with the cell, and known quantities of $InsP_3$ were photolytically released in the cytosol. Photorelease of ~70 μM $InsP_3$ was ineffective in producing a detectable calcium transient in the soma and proximal dendrites of five of seven cells tested. In the remaining two cells, a small and slowly rising calcium transient was observed after photorelease of $InsP_3$. The response of one of these neurons is shown in Fig. 1 A. Depolarization of the same neuron to 0 mV for 400 ms evoked a 10-fold larger Fluo-3 $\Delta F/F$ signal. The age of the cells, 10–16 d in vitro, was chosen because studies of long-term depression in cultured Purkinje neurons have typically used this range. We also examined a few neurons at 5–7 d in vitro since it has been shown that the percentage of Purkinje neurons with glutamate metabotropic receptor–evoked calcium release peaks at this time (Yuzaki and Mikoshiba, 1992). Only one of the four Purkinje neurons examined at 5–7 d in vitro showed an $InsP_3$-evoked calcium release. As with older neurons, the amplitude of the $InsP_3$-evoked calcium transient in the cell that showed a response was small, with a time-to-peak of several seconds. In all cultured Purkinje cells that showed an $InsP_3$-evoked calcium transient, the fluorescence increase after photorelease of $InsP_3$ was markedly smaller than that obtained with a 200-ms depolarization of the neurons to 0 mV.

An upper estimate of the rate of release of calcium with $InsP_3$ can be made in the few cultured Purkinje cells that demonstrated an $InsP_3$-evoked calcium transient. Since the affinity of Fluo-3 for calcium is ~450 nM, the dye will be saturated at calcium concentrations greater than several micromolar. The amplitudes of the $\Delta F/F$ Fluo-3 signals after photorelease of $InsP_3$ in cultured Purkinje cells were much smaller than the amplitudes of the calcium transients produced by a 200- or 400-ms depolarization of the cell to 0 mV so it can be safely assumed that the indicator was not saturated during the $InsP_3$-evoked calcium transients. Therefore, the concentration of calcium reached during the peak of the $InsP_3$-evoked calcium responses is at the very most a few micromolar. Considering the slow rate of rise of the $InsP_3$-evoked transients in cultured cells, the maximum rate of calcium release (in the few cells which did have a response) is estimated to be 0.1–5 $\mu M \cdot s^{-1}$.

Most experiments studying the properties of $InsP_3$-evoked calcium transients in Purkinje cells have used rat cerebellar slices. We ascertained that the properties of $InsP_3$-evoked calcium transients in acutely prepared mouse Purkinje cells are like those described in rat Purkinje cells by performing similar experiments in freshly dissociated Purkinje cells from young mice. We chose dissociated neurons over Purkinje cells in slices to improve the space clamp and reduce the time taken to equilibrate the patch pipette contents with the cytosol. As in rat Purkinje cells, intracellular photorelease of >10 μM $InsP_3$ increased calcium in mouse-dissociated Purkinje cells (Fig. 1 B). The calcium transient shown in Fig. 1 B, evoked by release of ~70 μM $InsP_3$, peaked within 80 ms. This response is typical of all those observed in mouse-dissociated Purkinje neurons (n = 7), and demonstrates that properties of $InsP_3$-evoked calcium transients in mouse Purkinje cells are

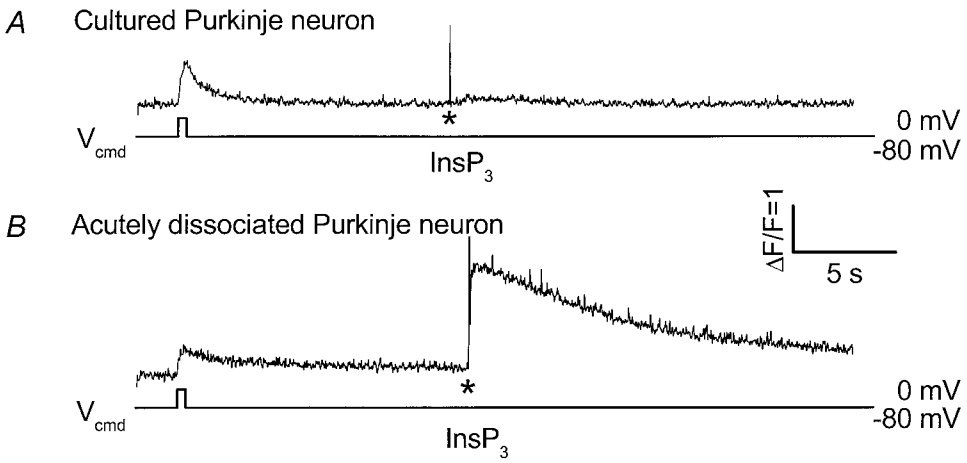

Figure 1. $InsP_3$-evoked calcium release is impaired in Purkinje cells maintained in culture. (A) A Purkinje cell, maintained for 13 d in culture, was whole-cell voltage clamped with a pipette containing 150 μM caged $InsP_3$ and 200 μM of the calcium indicator Fluo-3. The fluorescence emitted by Fluo-3 is calcium dependent, and an increase in the $\Delta F/F$ signal presented here indicates an increase in the intracellular cytosolic free calcium concentration. Depolarization of the cell to 0 mV for 400 ms produced a rapid transient increase in $[Ca^{2+}]_i$. In contrast, photorelease of ~70 μM $InsP_3$ (*) produced only a very small and slow increase in $[Ca^{2+}]_i$. Experiment FEB1999A. (B) A Purkinje cell, freshly dissociated from the cerebellum of a 12-d-old mouse, was whole-cell voltage clamped on the same setup using the same solutions. Photorelease of ~70 μM $InsP_3$ (*) produced a large and fast calcium transient. The amplitude of the $InsP_3$-evoked calcium transient was substantially larger than that produced by depolarization of the cell. Experiment FEB0599A.

similar to those in rat Purkinje cells (Khodakhah and Ogden, 1995). In contrast to cultured Purkinje cells, in dissociated Purkinje cells the amplitudes of the $InsP_3$-evoked calcium transients were severalfold larger than that of the depolarization-induced calcium rise.

Ryanodine receptors are present throughout Purkinje cells (Ellisman et al., 1990), except in the dendritic spines, and share a common calcium pool with $InsP_3$ receptors (Khodakhah and Armstrong, 1997b). We examined the ability of caffeine in releasing calcium from internal calcium stores via ryanodine receptors. Similar to $InsP_3$-evoked responses, caffeine-evoked calcium transients were significantly arrested in cultured Purkinje cells. Four of six neurons challenged with 15 mM caffeine showed a small calcium transient. One of these responses is presented in Fig. 2. Application of caffeine (Fig. 2 A) resulted in a very small change in the emitted fluorescence, much smaller than that seen with depolarization of the same cell to 0 mV for 400 ms (Fig. 2 B). In contrast, in all the acutely dissociated neurons tested ($n = 4$), caffeine evoked responses (Fig. 2 C) that were larger than those evoked by a 400-ms depolarization to 0 mV (Fig. 2 D).

The average $InsP_3$-evoked Fluo-3 $\Delta F/F$ was 3.3 ± 0.7 (SEM, $n = 7$), 40-fold larger than the same in cultured Purkinje cells (0.08 ± 0.07, SEM, $n = 10$). We also calculated the mean of the $InsP_3$- or caffeine-evoked responses, normalized to peak calcium transients induced by 200-ms depolarizations to 0 mV for all cultured and dissociated Purkinje cells (Fig. 3). The average normalized $InsP_3$-evoked transient in the dissociated neurons was over 40-fold larger than in cultured neurons, and that of the caffeine-evoked response was 15-fold larger.

We postulated that the lack of prominent $InsP_3$- and caffeine-evoked calcium transients in cultured Purkinje cells was a result of depleted calcium stores. Rapid depletion of calcium stores has been shown to occur in cells maintained in primary cultures (Murphy and Miller, 1988; Brorson et al., 1991). To test this possibility, we increased the cytosolic free calcium concentration by brief depolarization of the cell to activate voltage-dependent calcium channels and allow calcium influx. In acutely prepared Purkinje cells, this procedure has been shown to partially refill the stores (Khodakhah and Armstrong, 1997b). The efficacy of $InsP_3$ and caffeine in mobilizing calcium was then tested soon after depolarization in three neurons (six trials). Refilling the stores failed to rescue $InsP_3$- or caffeine-evoked responses in cultured Purkinje cells as shown in Fig. 4.

DISCUSSION

We directly examined the properties of calcium stores in cultured and acutely prepared mouse Purkinje cells by intracellular photolytic release of $InsP_3$, and by bath

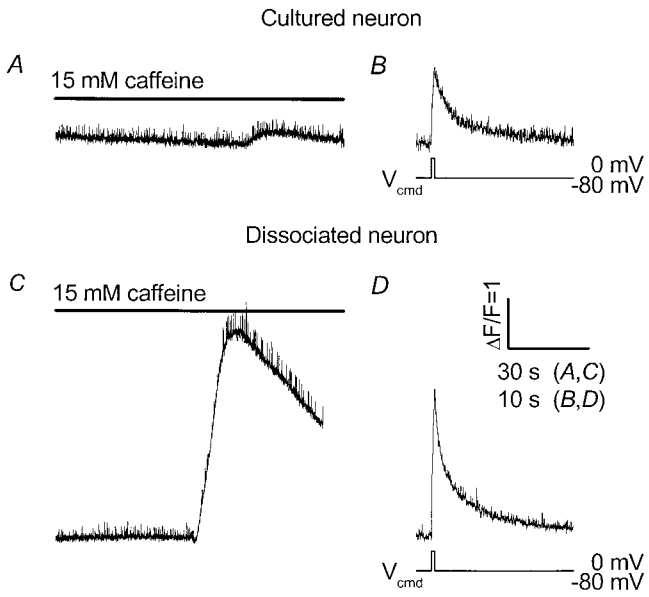

Figure 2. Caffeine-evoked calcium release is impaired in cultured Purkinje cells. (A) Shown is the efficacy of 15 mM caffeine in mobilizing calcium from the intracellular stores in a voltage-clamped cultured Purkinje cell. The caffeine-evoked Fluo-3 $\Delta F/F$ signal was smaller than the transient produced by depolarization of the cell to 0 mV for 400 ms (B). Experiment FEB2499D. (C) Application of 15 mM caffeine to a dissociated Purkinje cell resulted in a large calcium transient. The amplitude of the caffeine-evoked calcium transient was larger than that produced by depolarization of the cell to 0 mV for 400 ms (D). Experiment FEB0399B.

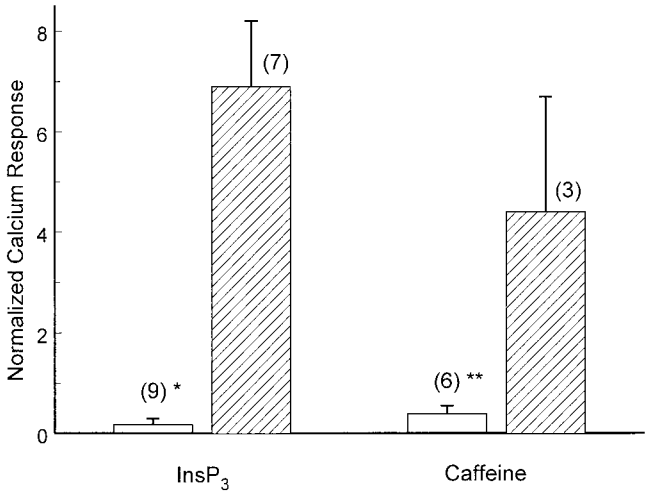

Figure 3. Comparison of normalized $InsP_3$- and caffeine-evoked calcium release in freshly dissociated and cultured Purkinje cells. To compare $InsP_3$-evoked (>70 μM) and caffeine-evoked (15 mM) responses in different cells, we normalized each agonist-evoked response to a depolarization-evoked response in the same cell. Shown is the average (±SEM) of ratio of the peak Fluo-3 $\Delta F/F$ transients evoked by each agonist to the peak Fluo-3 $\Delta F/F$ transient resulting from a 200-ms depolarization of the same neurons to 0 mV for freshly dissociated (crossed bars) and cultured (empty bars) Purkinje cells. Calcium release is clearly arrested in cultured neurons (*$P < 0.001$, **$P < 0.05$ by one way ANOVA).

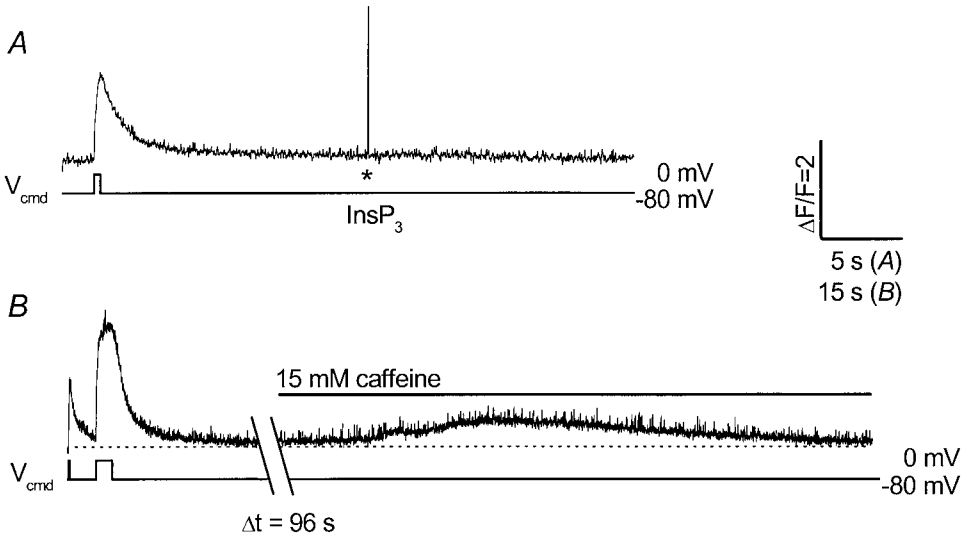

Figure 4. A procedure designed to refill calcium stores does not rescue $InsP_3$- or caffeine-evoked calcium release in cultured Purkinje cells. (A) The cytosolic free calcium concentration was transiently elevated by depolarization of a voltage-clamped cultured Purkinje cell to refill the stores. After the depolarization, photolytic release of $InsP_3$ (* ~70 μM), was ineffective in mobilizing calcium. (B) Similarly, attempts to refill the calcium stores did not increase the amplitude of a caffeine-evoked calcium transient in the same cell. Depolarization-evoked calcium transients were persistently larger than the caffeine-evoked responses in cultured Purkinje cells. Experiment FEB1999B.

application of caffeine. We find that both $InsP_3$ and ryanodine receptor–mediated calcium release are severely impaired in cultured Purkinje cells. 70% of the cultured Purkinje cells tested showed no $InsP_3$-evoked calcium transient with as much as 100 μM $InsP_3$. This is in marked contrast to acutely prepared Purkinje cells where all cells challenged with >10 μM $InsP_3$ responded with a large and rapidly rising transient. The maximal rate of calcium release in cultured Purkinje cells is estimated to be 0.1–5 $\mu M \cdot s^{-1}$, three to four orders of magnitude less than that found in acutely prepared Purkinje cells (as much as 1500 $\mu M \cdot s^{-1}$) (Khodakhah and Ogden, 1995).

There are several reports of intracellular calcium release in cultured Purkinje cells in response to activation of metabotropic glutamate receptors. In these studies, where calcium increases are observed, they are small (Yuzaki and Mikoshiba, 1992), slow to peak (Linden et al., 1994), with <20% of Purkinje cells responding by 10 d in culture (Brorson et al., 1991; Yuzaki and Mikoshiba, 1992). The properties of calcium release in cultured Purkinje cells has been assumed to be the same as that which occurs in situ. This study provides the first direct comparison between calcium release properties in cultured Purkinje cells with those acutely in prepared cells. Our results provide clear evidence that calcium release in cultured Purkinje cells is substantially arrested compared with that in vivo.

In these studies we examined $InsP_3$-evoked calcium release in the soma and proximal dendrites of cultured Purkinje neurons, and compared them with responses in the soma of acutely dissociated cells. It is possible that cultured Purkinje cells have prominent $InsP_3$-evoked responses in their distal dendrites that we missed. However, in cultured Purkinje neurons, labeling with $InsP_3$ receptor-specific antibodies suggests that $InsP_3$ receptors are evenly distributed throughout the Purkinje cell, including the somata and fine dendrites (Brorson et al., 1991; Yuzaki and Mikoshiba, 1992). Moreover, in Purkinje neurons in slices, $InsP_3$-evoked calcium release in cell bodies and dendrites are similar.

While caffeine mobilized calcium in all the acutely prepared Purkinje cells tested in this study, we find that it is less potent in cultured Purkinje cells. Our results are in agreement with the finding that caffeine-evoked responses in cultured Purkinje neurons are quite labile (Brorson et al., 1991; Yuzaki and Mikoshiba, 1992). Interestingly, even the diminished caffeine-sensitive calcium release has been shown to be required for potentiation of inhibitory postsynaptic currents (Hashimoto et al., 1996), and for culture-LTD in cultured Purkinje cells (Kohda et al., 1995; Inoue et al., 1998).

The reason for the impaired calcium release in Purkinje cells maintained in culture is not clear. Although $InsP_3$ and ryanodine receptors are present in both the cell bodies and dendrites of cultured Purkinje cells (Brorson et al., 1991; Yuzaki and Mikoshiba, 1992), some of these receptors may be nonfunctional, or may be present at low densities. It has also been demonstrated that type 1 $InsP_3$ receptors are avidly degraded in culture (Oberdorf et al., 1997), and it may be that the rate of degradation of type 1 $InsP_3$ receptors in culture is substantially accelerated compared with the same in vivo. Alternatively, Purkinje cells in culture may not be able to maintain a substantial calcium store. It is unlikely that the impaired calcium release reported here in culture is specific to Purkinje cells. Photorelease of as much as 40 μM $InsP_3$ is ineffective in mobilizing calcium in hippocampal and striatal neurons in primary cultures (Khodakhah and Ogden, 1993), cells that are shown to

express $InsP_3$ receptors in situ (Worley et al., 1989). Alteration in intracellular calcium release properties when cells are maintained in culture, therefore, may be a finding relevant to many different cells. Without doubt, the changes in the second messenger pathways in culture will be critically dependent on the culture conditions and there may be a culture condition that faithfully preserves the in vivo physiological properties of the cells. The culture conditions used here were chosen to mimic those used in the studies of culture-LTD.

This study was prompted by the discrepancy in the data obtained regarding the role of $InsP_3$-evoked calcium release in the induction of LTD in Purkinje cells in acutely prepared slices, and those maintained in culture (Kasono and Hirano, 1995; Narasimhan et al., 1998). While in cerebellar slices $InsP_3$-evoked calcium release seems to be necessary and sufficient to induce LTD (Khodakhah and Armstrong, 1997a; Finch and Augustine, 1998; Inoue et al., 1998), in cultured Purkinje cells $InsP_3$-evoked calcium release is thought not to be required for the induction of culture-LTD (Narasimhan et al., 1998). The culture conditions used here are the same as that in the later study, and the reason for lack of involvement of $InsP_3$-receptors in culture-LTD in the studies reported by Narasimhan et al. (1998) is likely to be the consequence of impaired calcium release reported here. In a separate study using different culture conditions, $InsP_3$ is reported to be effective in inducing culture-LTD only if it is accompanied with coactivation of AMPA receptors (Kasono and Hirano, 1995). The need for coactivation of AMPA receptors in culture may also be directly a consequence of impaired $InsP_3$-evoked calcium release in cultured Purkinje cells. Sodium influx through the AMPA receptors is thought to act on the Na^+–Ca^{2+} exchanger to slow calcium efflux and thereby increase $[Ca^{2+}]_i$ (Linden, 1994). Given the reduced calcium release in cultured Purkinje cells, the additional boost in the $[Ca^{2+}]_i$ by the slowing of the exchanger may be necessary for the induction of culture-LTD by $InsP_3$.

Despite impaired calcium release, it is interesting that a form of plasticity is observed in cultured Purkinje cells. Work from many laboratories has demonstrated that several intracellular second messengers contribute to culture-LTD. These include nitric oxide and cyclic GMP (Linden et al., 1995) and diacylglycerol-stimulated increases in protein kinase C activity (Linden and Connor, 1991). Some of these pathways have also been implicated in the induction of LTD in slices (Crepel and Krupa, 1988; Daniel et al., 1993; Boxall and Garthwaite, 1996; Lev-Ram et al., 1997). It is plausible that, despite the requirement for functional $InsP_3$ receptors, under physiological conditions long-term depression may be mediated by coactivation of several different second messenger pathways. The relative contribution of each of these pathways may be modulated physiologically in vivo as it appears to be in vitro.

The use of culture-LTD as a model for cerebellar long-term depression is based on the assumption that it shares the same fundamental mechanisms as cerebellar LTD. While minor differences between acutely prepared and cultured Purkinje cells have been described previously (summarized in Linden, 1996), our results provide direct evidence that one of the second messenger systems that is thought to be necessary for LTD in cerebellar slices is significantly altered in cultured Purkinje cells.

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