Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4976–4980, June 1993
Neurobiology

Functional heterogeneity of calcium release by inositol trisphosphate in single Purkinje neurones, cultured cerebellar astrocytes, and peripheral tissues

(flash photolysis)

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Communicated by Arnold Burgen, February 26, 1993

ABSTRACT **Purkinje neurones of the cerebellar cortex are rich in receptors for the Ca-mobilizing second messenger inositol trisphosphate ($InsP_3$) in association with intracellular Ca stores. Cytosolic Ca ions are important in regulating neuronal excitability but it has proved difficult to demonstrate $InsP_3$-evoked release of Ca in mammalian central neurones directly. Intracellular release of $InsP_3$ by flash photolysis of caged $InsP_3$, combined with whole-cell patch clamp and microspectrofluorimetry of Ca indicators, allows comparison of $InsP_3$-evoked Ca release in single Purkinje cells in cerebellar slices with the same process in cultured astrocytes and peripheral tissues. In astrocytes, hepatocytes, exocrine cells, and vascular endothelium, minimal Ca release from stores requires photorelease of $InsP_3$ at concentrations of 0.2–0.5 μM, and maximal efflux as judged by the rate of increase of Ca concentration is seen with 5–10 μM $InsP_3$. In contrast in Purkinje cells, $InsP_3$ concentrations of $\geq$9 μM were required to produce minimal Ca release from stores under the same conditions, and Ca efflux increased with $InsP_3$ concentrations up to 70–80 μM. Furthermore, the rate of increase and size of the Ca concentration in Purkinje cells are 10- to 30-fold greater than in astrocytes and peripheral tissues. The $InsP_3$ sensitivity was not affected by changing exogenous cytosolic Ca buffering, suggesting that endogenous Ca binding cannot account for the difference. The results show a functional difference in $InsP_3$-evoked Ca release between Purkinje cells and peripheral tissues.**

Purkinje neurones of the cerebellar cortex contain a particularly high density of binding sites for the Ca-mobilizing second messenger inositol trisphosphate ($InsP_3$) (1) but, unlike peripheral tissues, Ca release from intracellular stores by $InsP_3$ applied in the cytosol has proved difficult to demonstrate in Purkinje cells. The cytosolic free Ca^{2+} concentration is important in regulating the excitability of Purkinje cells and other mammalian central neurones and it is thought that release of Ca^{2+} from intracellular stores by "metabotropic" actions of neurotransmitters is an important means of controlling neuronal responsiveness in the short and long term (2, 3). In peripheral nonexcitable tissues, quantitative time-resolved studies of $InsP_3$-evoked Ca release from stores have been made by rapid mixing in permeabilized cells or by flash photolysis of caged $InsP_3$ in single cells. However, present evidence for $InsP_3$-evoked Ca release in central neurones is less direct and does not permit quantitative comparisons. In the experiments described here, rapid release of $InsP_3$ from caged $InsP_3$ by flash photolysis in the cytosol of Purkinje cells in rat cerebellar slices produced large fast increases of cytosolic Ca concentration. The characteristics of this process are compared with data obtained in the same way in tissue-cultured astrocytes and peripheral tissues. The results show that in Purkinje neurones Ca efflux from stores was activated at higher $InsP_3$ concentrations and was much faster than in peripheral tissues or astrocytes.

METHODS

The whole-cell patch clamp technique (4) was used for electrical recording and to introduce Ca indicator and caged $InsP_3$ [P^4-2-nitrophenylethyl ester of *myo*-D-inositol 1,4,5-trisphosphate (5)] into the cytosol of Purkinje neurones in thin cerebellar slices (6) from 12- to 22-day-old rats or cerebellar astrocytes in primary culture. Normal external solution contained 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1.25 mM $NaHPO_4$, 25 mM $NaHCO_3$, and 11 mM glucose (pH 7.4) in an atmosphere of 95% O_2/5% CO_2. Normal internal solution contained 153 mM potassium gluconate, 8 mM Hepes (pH 7.2), 3 mM $MgSO_4$, 3 mM Na_2 ATP, 200 μM fluo-3 (Molecular Probes) or 500 μM furaptra (Molecular Probes), plus caged $InsP_3$. Experiments were made at room temperature, 22–25°C. Microspectrofluorimetry via a ×40 0.75 NA water-immersion objective was from an area, usually just the soma, defined by a rectangular diaphragm in an image plane of the microscope, viewed by a TV camera and photomultiplier. Fluorescence data are shown integrated with a 5-ms time constant. The excitation light intensity, Ca indicator concentrations, and area of collection of emitted light were kept the same from cell to cell to ensure that the amount of indicator and caged $InsP_3$ loaded and the initial Ca concentration were reproducible. Flash photolysis was produced by the arc of a xenon flashlamp (7) focused into the slice via a silica condenser and UG11 band-pass filter (300–350 nm). An extinction coefficient for cerebellar slices of 10 cm^{-1} was measured at 320 nm, giving 26% attenuation of the flash in a 300-μm slice; slice thickness was measured in each experiment by calibrated focusing. Control experiments for the byproducts released by photolysis were made with photolysis of 2-nitrophenylethyl ester of inorganic phosphate. Calibration of photolysis in the microscope over the area visualized in an experiment was by titration of the fluorescence change produced in the pH dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by protons released stoichiometrically during photolysis of caged MgATP (8); full-lamp output of ≈100 mJ in 1 ms at 300–350 nm produced 52% photolysis of caged ATP and produces equivalent conversion of caged $InsP_3$ (5). Lamp discharge energy and cage concentration were altered to change the extent of photolysis and the $InsP_3$ concentration released as required. Reproducibility of photolysis within the same cell at the same lamp discharge settings was estimated as 10% (standard deviation);

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Abbreviation: $InsP_3$, inositol trisphosphate.

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between experiments, including differences due to loading of caged $InsP_3$, lamp alignment, and true cell–cell variation, the errors are more difficult to estimate but at low $InsP_3$ concentrations the standard deviation of experimental data is <50% in terms of concentration.

RESULTS

Flash photolytic release of $InsP_3$ from caged $InsP_3$ (5, 9–12) has been used in peripheral tissues to study the kinetics of $InsP_3$-evoked Ca release. A pulse of $InsP_3$ is released with a halftime of 3 ms at known concentration in the cytosol adjacent to the Ca stores, overcoming problems of slow access and avid metabolic breakdown, which otherwise distort both the kinetics and concentration of $InsP_3$ achieved at the release site. Photorelease of $InsP_3$ to give cytosolic concentrations of 0.2–0.5 μM in smooth muscle (9), liver (10), exocrine cells (11), and endothelial cells (12) produces a slow increase of cytosolic Ca concentration. Maximal Ca efflux, judged by the minimum latency and maximum rate of increase of free Ca concentration, is at 5–10 μM $InsP_3$. Experiments made in primary cultures of cerebellar astrocytes show that Ca release from stores occurs at the same $InsP_3$ concentrations observed in nonexcitable peripheral tissues. Fig. 1*A* shows the fluorescence increase produced when $InsP_3$ was released from caged $InsP_3$ by a 1-ms pulse of near UV radiation in a cerebellar astrocyte loaded in a whole-cell patch clamp configuration with the fluorescent Ca indicator fluo-3 at 200 μM and 5 μM caged $InsP_3$. A low-intensity flash releasing 0.3 μM $InsP_3$ produced a slow increase in fluorescence after a 300-ms delay and a more intense flash in the same cell, releasing 0.7 μM, produced a faster rise of fluorescence due to Ca after a 130-ms delay. Inositol phosphate turnover and Ca release from stores are produced in primary astrocyte cultures by neurotransmitters (13, 14), including L-glutamate, and this observation of $InsP_3$-evoked Ca release confirms an action of $InsP_3$ on Ca stores in astrocytes similar to that documented for peripheral tissues.

In contrast to the results with astrocytes and peripheral tissues, when recordings were made from primary cultures of hippocampal and striatal neurones, Ca release from stores could not be elicited by photolytic release of $InsP_3$ up to 40 μM, under the same experimental conditions and even in the same cultures that produced responsive astrocytes. The underlying reason for the failure of $InsP_3$ to release Ca in tissue culture neurones is not known, but the possibility that it is due to changes that occur in culture was tested by making experiments in slices of central nervous system tissue.

Whole-cell recordings made in Purkinje cells in cerebellar slices also showed no Ca release with $InsP_3$ concentrations up to ≈9 μM. Fig. 1*B* shows a very small increase in fluo-3 fluorescence recorded from the soma of a Purkinje cell in which an estimated 9 μM $InsP_3$ was released by photolysis at the time indicated by the arrow. In some cells no change was seen. At higher concentrations [e.g., 19 μM in the same cell (Fig. 1*B*, lower trace)], a distinct fluorescence transient due to Ca mobilization was seen. Responses were not affected by removing external Ca (replaced by Mg^{2+} with 500 μM EGTA), conditions that prevented the increase of fluorescence due to Ca influx during prolonged depolarization (data not shown). The increase of cytosolic Ca concentration after

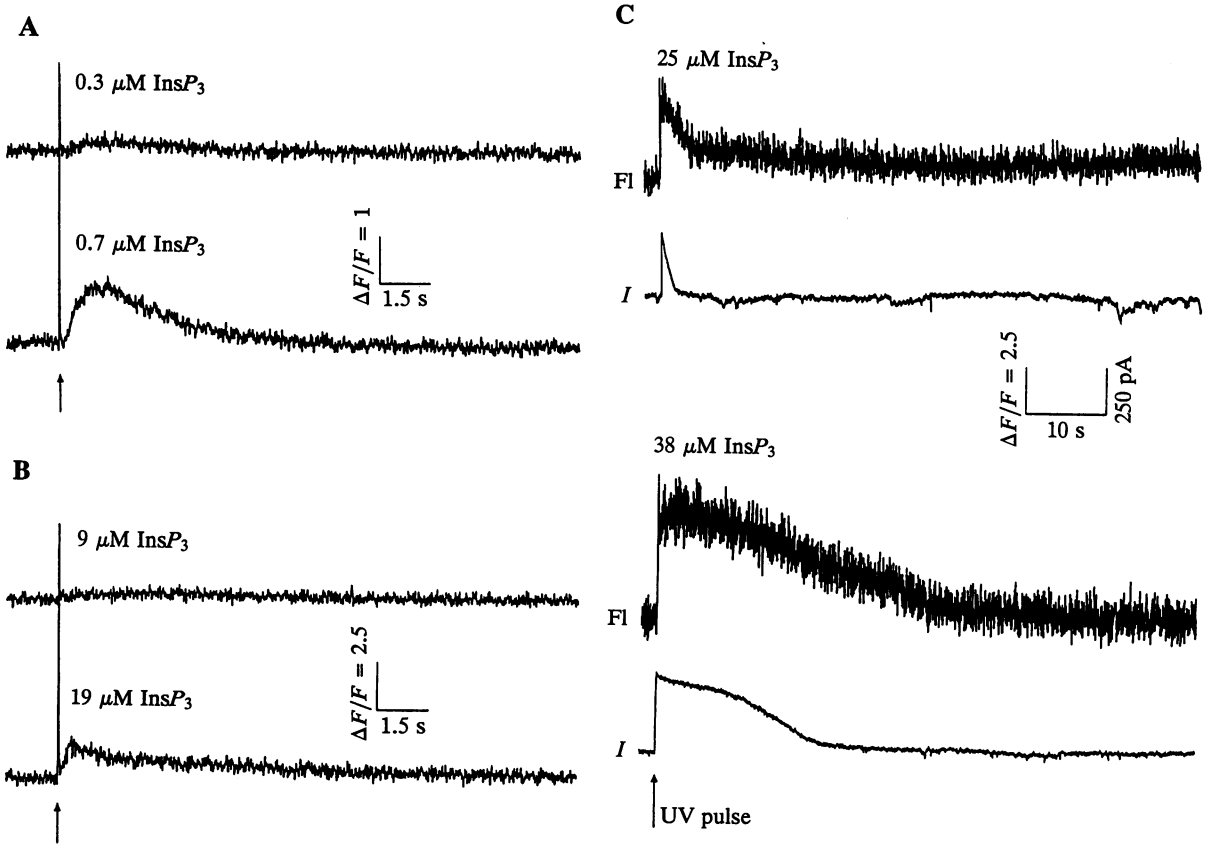

FIG. 1. Comparison of $InsP_3$-evoked Ca mobilization in cultured cerebellar astrocytes (*A*) (membrane potential, −50 mV) and cerebellar Purkinje neurones in slices (*B* and *C*) (clamp potential, −70 mV). Fluorescence increase of the Ca indicator fluo-3 (200 μM) after photolytic release of $InsP_3$ in the cytosol at the time indicated by the arrow is shown. Concentrations of $InsP_3$ released were 0.3 μM and 0.7 μM (same cell) (*A*) and 9 μM and 19 μM (same cell) (*B*). (*C*) Lower traces are membrane current (*I*) at −70 mV, showing activation of K^+ conductance. $InsP_3$ concentrations were 25 μM and 38 μM, same cell. The Ca-induced fluorescence change (ΔF) is expressed as a fraction of fluorescence (F) in the unstimulated cell. Fl, fluorescence.

release of 25 μM and 38 μM InsP_3 by flashes of different intensities in another cell is shown in Fig. 1*C*, which shows fluorescence due to fluo-3 (upper traces) and the membrane current at a membrane potential of −70 mV (lower traces). The fluorescence increase is accompanied by an outward current that had a reversal potential at −85 mV, the potassium equilibrium potential. To determine whether K^+ or Cl^- was the permeant ion, gluconate, the impermeant internal anion, was replaced with Cl^-, a procedure that had no effect on the reversal potential. Thus, the outward current was due to a conductance increase to K^+, perhaps due to the increased submembrane Ca concentration, and would be expected to depress excitability (see below).

The time course of fluorescence and conductance immediately after the 1-ms flash is shown in Fig. 2 (trace a = 38 μM and trace b = 19 μM; traces a and b are from one cell; trace c = 19 μM and trace d = 9 μM; traces c and d are from another cell). There is an initial artifact of 10–20 ms, present in control experiments without caged InsP_3 and mainly due to phosphorescence in the microscope optics induced by the flash. As the concentration of InsP_3 released by the flash was increased, the delay before the rise of fluorescence decreased from 76 ms at 9 μM to 16 ms at 38 μM, and at 76 μM it arose during the flash artifact (data not shown). The reduced delay and increased rate of rise of fluorescence show an increased Ca efflux from stores into the cytosol as InsP_3 concentration was increased. The outward current in the plasma membrane arose within a few milliseconds of the fluorescence, suggesting that it is activated by cytosolic Ca^{2+}. The delays may be compared with those reported in peripheral tissues. Delays at low concentrations of InsP_3, 0.1 μM–0.4 μM, were 150 ms in smooth muscle and 200–300 ms in liver and endothelial cells. At high concentrations, >10 μM InsP_3, Ca starts to increase within 20 ms (9–12).

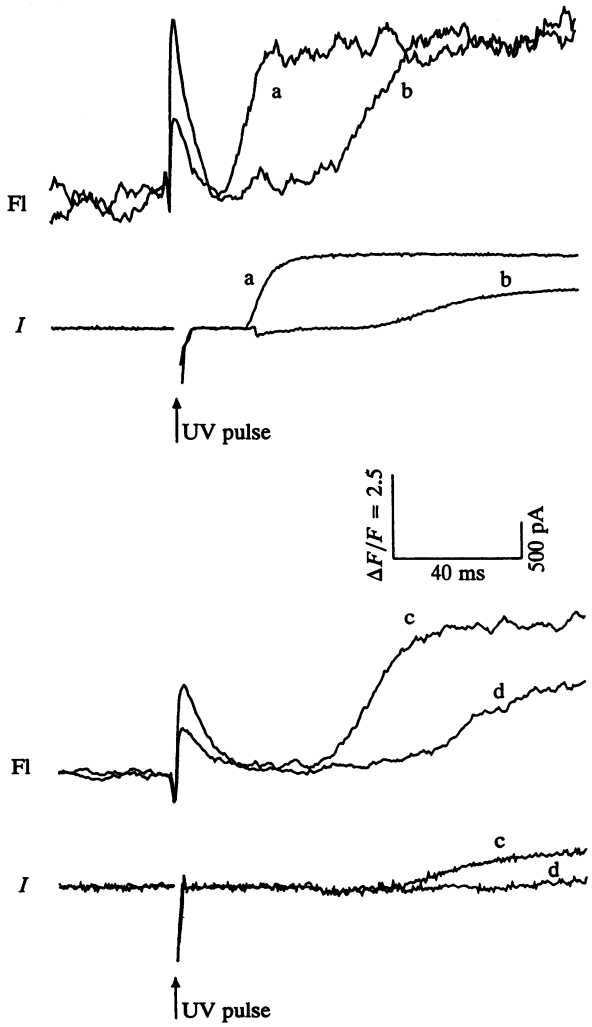

FIG. 2. Delays and rates of increase of Ca–fluo-3 fluorescence (Fl, upper traces) and outward current (*I*, lower traces) after photolytic release of a pulse of InsP_3. Traces: a, 38 μM InsP_3; b, 19 μM InsP_3; c, 19 μM InsP_3; d, 9 μM InsP_3. Traces a and b were from one cell; traces c and d were from another cell. After the flash, there was an artifact of 15- to 20-ms duration, due to phosphorescence in the optics, then fluorescence due to Ca release rose after 50 ms at low and 20 ms at high InsP_3 concentration. The membrane conductance increased within a few milliseconds after the increase of whole-cell Ca signal. The rate of increase of the Ca–fluo-3 signal increased at high InsP_3 concentration.

The immediate effect of InsP_3 release on the excitability of Purkinje cells is expected to be an inhibition resulting from activation of the K conductance. Fig. 3*A* shows the effects of photolytic release of InsP_3 during a current clamp recording of membrane potential. Current pulses applied to produce depolarization resulted in discharge of one full-size and one attenuated action potential at the beginning of the pulse. Ca release by 19 μM InsP_3 produced membrane hyperpolarization from −65 mV to −75 mV and a conductance increase, suppressing action potential discharge, which recovered when the Ca signal had subsided.

Fig. 3*B* shows an increase of fluo-3 fluorescence in the dendritic regions of a Purkinje cell evoked by release of InsP_3 (concentration 38 μM if caged InsP_3 reached equilibrium in the dendritic tree) when this area of the cell rather than the soma was seen by the photomultiplier tube, confirming a Ca-mobilizing action of InsP_3 in both soma and dendrites.

The data presented here show that >10-fold higher concentrations of InsP_3 are required to elicit Ca release in Purkinje cells when compared to astrocytes in primary culture or to peripheral tissues such as hepatocytes, exocrine cells, or vascular endothelia and that the Ca mobilization is faster and larger. Two possible explanations are that different isoforms (15–17) of the InsP_3-evoked Ca release channel are present in Purkinje cells and astrocytes or that the high Ca-buffering capacity thought to exist in the Purkinje cells may play a role (18). It is well documented that Ca and InsP_3 act as coagonists (19–21) at low free-Ca concentrations, up to ≈500 nM. If a cooperative action of Ca and InsP_3 is required for Ca release and Ca concentration is very tightly regulated, then high concentrations of photolytically released InsP_3 may be needed to initiate the process in Purkinje cells when compared to peripheral tissues. This point was tested by experiments in which the Ca buffering of the Purkinje cell was modified to a different extent by a high concentration (1.2 mM) of the high-affinity dye fluo-3 [K_d = 0.4 μM (22)] or by monitoring Ca concentration with the low-affinity dye furaptra at 500 μM [K_d = 44 μM (23)]. In the former case, the bound/free Ca would be ≈2000:1 at 0.1 μM free Ca, large compared with reported values for endogenous Ca buffering (30–75:1; refs. 24–26), and with furaptra the bound/free Ca would be 11:1, small relative to endogenous buffering. Fig. 4*A* shows fluorescence traces in a Purkinje cell loaded with 1.2 mM fluo-3 after photolytic release of 9 μM and 19 μM InsP_3. The concentration required to mobilize Ca was not altered even though the addition of this much mobile buffer might be expected to substantially distort Ca gradients in the cytosol.

Experiments were made with furaptra as the Ca indicator to minimize the effects of dye buffering and also to take advantage of the low affinity of furaptra for Ca^{2+} to provide a better estimate of the spatial average of Ca concentration, particularly when high local Ca transients are present during the early part of Ca efflux. The quenching of furaptra fluorescence (>490 nm) by Ca was readily calibrated when using excitation light of 400–440 nm to yield the free con-

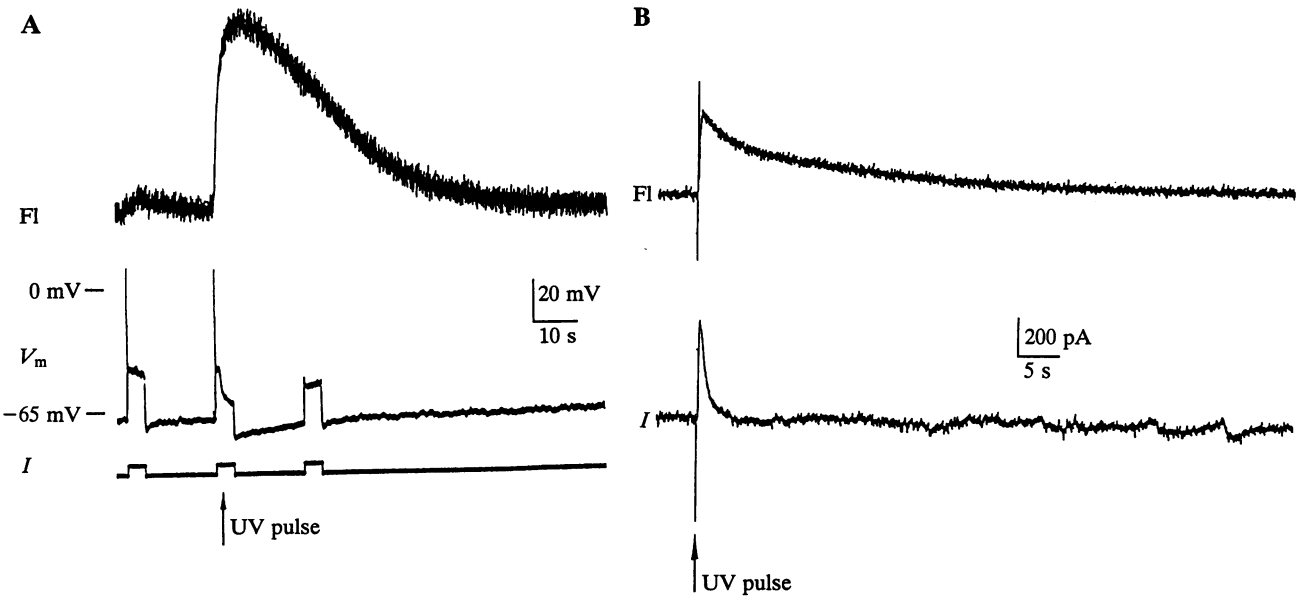

FIG. 3. (*A*) Current clamp recording of membrane potential (V_m, middle trace) in a Purkinje neurone. Depolarizations induced by 40-pA current pulses gave rise to one full-size and one attenuated action potential, accompanied by a small increase of Ca–fluo-3 fluorescence (Fl, upper trace). Photolytic release of 19 μM InsP_3 (arrow) 500 ms after the start of the current pulse produced a fluorescence increase accompanied by membrane hyperpolarization and conductance increase. The following current pulse failed to elicit action potentials, which recovered when tested 30 s later. (*B*) Ca release by InsP_3 in the dendrites of a Purkinje cell. The photomultiplier sees only the dendritic field of a neurone. InsP_3 released by a 1-ms full-intensity flash with 1.2 mM fluo-3 and 100 μM caged InsP_3 loaded by whole-cell patch clamp. Upper trace, fluo-3 fluorescence; lower trace, whole cell current at −70 mV recorded at the soma.

centration averaged over the soma (see ref. 23). Three traces from consecutive flashes in the same cell, digitized and converted pointwise to free cytosolic Ca concentration, are shown in Fig. 4*B*. The InsP_3 concentrations required for Ca release were as high with furaptra as with fluo-3, showing that the buffering inherent with high-affinity indicators such as fluo-3 was not responsible for the high InsP_3 concentrations required in Purkinje cells. Furthermore, the large range of Ca buffering used in these experiments (from 5:1 to 2000:1) and in experiments in peripheral tissues suggests that the endogenous Ca buffering is unlikely to be an important factor in determining the InsP_3 concentrations needed. This leads to the conclusion that the InsP_3 receptor itself may have different properties in the Purkinje cells with respect to astro-

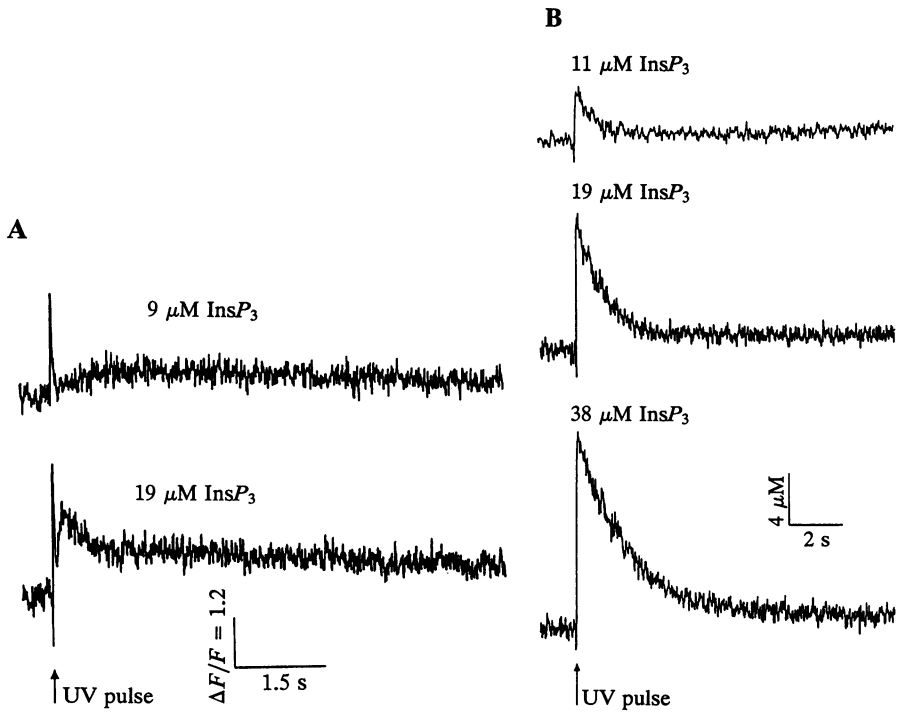

FIG. 4. (*A*) Fluo-3 fluorescence recorded from the soma of a Purkinje cell loaded with 1.2 mM fluo-3. Upper trace, release of 9 μM InsP_3; lower trace, release of 19 μM InsP_3. (*B*) Increase of cytosolic Ca concentration in a Purkinje soma monitored with 500 μM furaptra, with excitation at 400–440 nm and emission at >490 nm. InsP_3 at 11 μM, 19 μM, and 38 μM was released by consecutive flashes in the same cell. Ca concentration was calculated with K_d = 44 μM (see ref. 23) and the fluorescence in low and high Ca concentrations.

cytes and peripheral tissues. Indeed, a structural difference due to alternative splicing has been reported (15–17). Alternatively, it is possible that a high density of InsP_3 binding sites in Purkinje cells, perhaps inactive receptors, may rapidly reduce the concentration of InsP_3 adjacent to the membranes of the Ca stores and thereby require release of a higher concentration.

The better quantization and time resolution when furaptra was used as a Ca indicator show that very high Ca concentrations averaged over the soma are recorded (mean value 33 μM in 18 cells at 38 μM InsP_3), much larger than the peak Ca concentrations seen in peripheral tissues. Furthermore, the times to peak Ca at low (9 μM) and high (38 μM) InsP_3 of 143 ms and 35 ms are much shorter than those in liver, smooth muscle, and endothelial cells. Thus, the rate of change of free Ca concentration, and therefore the efflux from stores, is very large in Purkinje cells, $\approx$1 μM·ms^{-1} at 38 μM InsP_3, relative to the maximum rate in peripheral tissues of $\approx$0.03 μM·ms^{-1}. These differences are likely to be due to the specialized structures, such as high densities of InsP_3 receptors, Ca stores, and Ca binding proteins, present in Purkinje cells. These structural features, the requirement for a high InsP_3 concentration, and the large Ca efflux suggest an adaptation of InsP_3-mediated signaling in Purkinje neurones when compared to other tissues.

We thank David Trentham for providing caged InsP_3 used in these experiments and for discussion. This work was supported by the Medical Research Council and a Glaxo Scholarship.

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