Spontaneously active and $InsP_3$-activated ion channels in cell nuclei from rat cerebellar Purkinje and granule neurones

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Increases in Ca^{2+} concentration in the nucleus of neurones modulate gene transcription and may be involved in activity-dependent long-term plasticity, apoptosis, and neurotoxicity. Little is currently known about the regulation of Ca^{2+} in the nuclei of neurones. Investigation of neuronal nuclei is hampered by the cellular heterogeneity of the brain where neurones comprise no more than 10% of the cells. The situation is further complicated by large differences in properties of different neurones. Here we report a method for isolating nuclei from identified central neurones. We employed this technique to study nuclei from rat cerebellar Purkinje and granule neurones. Patch-clamp recording from the nuclear membrane of Purkinje neurones revealed numerous large-conductance channels selective for monovalent cations. The nuclear membrane of Purkinje neurones also contained multiple $InsP_3$- activated ion channels localized exclusively in the inner nuclear membrane with their receptor loci facing the nucleoplasm. In contrast, the nuclear membrane of granule neurones contained only a small number of mainly anion channels. Nuclear $InsP_3$ receptors ($InsP_3Rs$) were activated by $InsP_3$ with $EC_{50} = 0.67\ \mu M$ and a Hill coefficient of 2.5. Ca^{2+} exhibited a biphasic effect on the receptors elevating its activity at low concentrations and inhibiting it at micromolar concentrations. $InsP_3$ in saturating concentrations did not prevent the inhibitory effect of Ca^{2+}, but strongly increased $InsP_3R$ activity at resting Ca^{2+} concentrations. These data are the first evidence for the presence of intranuclear sources of Ca^{2+} in neurones. Ca^{2+} release from the nuclear envelope may amplify Ca^{2+} transients penetrating the nucleus from the cytoplasm or generate Ca^{2+} transients in the nucleus independently of the cytoplasm.

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Synaptic activity can evoke a rise in Ca^{2+} concentration in the nucleus of neurones either by generating a regenerative Ca^{2+} wave, spreading from activated synapses to the cell body or by depolarizing the neurone and activating voltage-operating Ca^{2+} channels in the somatic plasma membrane (Berridge, 1998; Power & Sah, 2002). Increases in Ca^{2+} concentration in neuronal nuclei regulate gene transcription by activating nuclear Ca^{2+}-sensitive kinases and phosphatases or by directly affecting Ca^{2+}-binding transcription factors. The two regulatory mechanisms may be exemplified by the transcription factors cAMP responsive element binding protein (CREB) and downstream response element-antagonist modulator (DREAM), respectively (Hardingham *et al.* 2001; West *et al.* 2002). Nuclear Ca^{2+} transients have been implicated in a number of physiological phenomena including neuronal development, survival, and activity-dependent long-term neuronal plasticity (Berridge, 1998; Bading, 2000; West *et al.* 2002).

Little is currently known about Ca^{2+} regulation in the nuclei of neurones. In non-neuronal cells this issue has been addressed in numerous studies, which have yielded conflicting results (reviewed in Santella & Carafoli, 1997; Bootman *et al.* 2000). There are two potential sources of Ca^{2+} that may generate an increase inside the nucleus. Ca^{2+} ions can penetrate the nucleus from the cytoplasm through numerous nuclear pores or they can be released into the nucleoplasm from the nuclear envelope. The nuclear envelope is a flattened cistern morphologically and biogenetically related to the endoplasmic reticulum. It has been shown that the nuclear envelopes of *Xenopus* oocytes, hepatocytes and a number of other cells are functional

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Ca^{2+} stores (Nicotera *et al.* 1990; Gerasimenko *et al.* 1995; Stehno-Bittel *et al.* 1995). The role of the nuclear envelope in the regulation of nuclear Ca^{2+} is the main issue of the current controversy. The reason for this controversy may at least partly result from different mechanisms of Ca^{2+} regulation in the nuclei of different cells.

One of the difficulties in studying neuronal nuclei is the heterogeneous cellular composition of the nervous system. Neurones constitute no more than 10% of the total number of brain cells (Kendal *et al.* 2000). The standard method of nucleus isolation includes homogenization of a tissue and subsequent differential centrifugation of the homogenate (Blobel & Potter, 1966). When applied to nervous tissue this method yields a mixture where neuronal nuclei comprise a minority group. Isolation of nuclei from certain parts of the brain, such as the cerebral or cerebellar cortex, can enrich the preparation with neuronal nuclei, but does not solve the problem. Further complication arises from the extreme diversity of the neurones themselves.

We have developed a method for isolating nuclei from identified central neurones. Using this technique we have studied ion channels in the nuclei of cerebellar Purkinje and granule neurones and found that they have different properties.

Methods

Isolation of neuronal nuclei

All experimental procedures involving animals and their care were conducted in conformity with the Animals (Scientific Procedures) Act 1986 and the guidelines of the Ministry of Public Health of Ukraine. Male Wistar rats (3- to 4-weeks old) were anaesthetized with ether and decapitated. The cerebellum was rapidly removed and placed in ice-cold (1–3°C) artificial cerebrospinal fluid (ACSF), containing (mM): NaCl 120, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 1, NaH_2PO_4 1.3, $NaHCO_3$ 26, glucose 10 and equilibrated with 95% O_2 and 5% CO_2. Coronal cerebellar slices (300 μm; see Fig. S1 of the Supplemental material available with this article online) were cut by hand or using a Vibratome (Campden Instruments, Sileby, UK). Slices were placed in a chamber on the stage of a Leica DMLFS upright microscope (Leica Microsystems, Germany) and continuously perfused with ACSF continuously oxygenated with 95% O_2 and 5% CO_2 at room temperature. Neurones were visualized using a × 40 long working distance water-immersion objective and a VX 45 Microscope CCD camera (PCO Computer Optics, Kelheim, Germany) with DIC/infrared optics.

Nuclei were collected from neurones with glass pipettes (Fig. 1*A*). The opening of the pipette varied depending on the size of the nucleus and was 20–30% larger than the nucleus diameter. The cytoplasm from a Purkinje or granule neurone was aspirated under visual control into the pipette until the nucleus was sucked into the pipette. This procedure was applied to several other nuclei until the collected nuclei with remains of the cytoplasm were ejected into a microtube of homogenization solution containing (mM): potassium gluconate 150, Hepes-KOH 10 (pH 7.3). Protease Inhibitor Cocktail (Roche Diagnostics UK Ltd) was added to the solution according to the manufacturer's instructions. The nuclei were cleared from remains of the cytoplasm by four to five strokes in a 2 ml Dounce tissue homogenizer (Bellco Glass, Vineland, NJ, USA). Comparison of nuclei isolated from Purkinje neurones (Fig. 1*B*) with nuclei

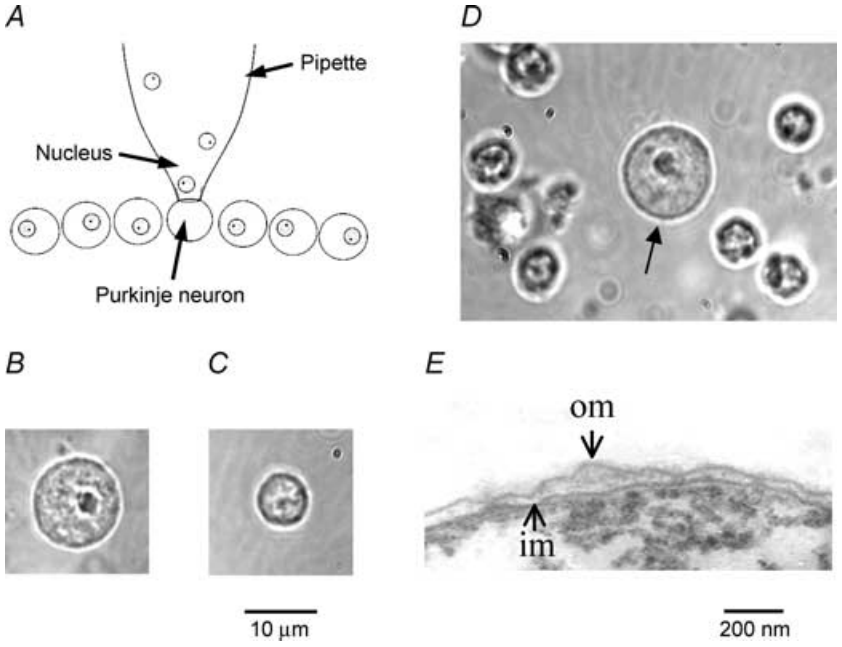

Figure 1. Isolated nuclei from neurones of rat cerebellar cortex
A, the method of isolating nuclei from central neurones. The cytoplasm from a neurone (here a Purkinje neurone) in a brain slice was aspirated into a glass micropipette with a relatively large opening until the nucleus was sucked into the pipette. The same pipette was used to collect nuclei from several neurones. *B* and *C*, individual nuclei isolated from a Purkinje neurone (*B*) and a granule neurone (*C*). *D*, a rough homogenate of the cerebellar cortex. The large nucleus from a Purkinje neurone (arrow) clearly differs from all other nuclei in the homogenate. *E*, a transmission electron micrograph of the periphery of the nucleus isolated from a Purkinje neurone. The nucleus had well-preserved inner (im) and outer (om) nuclear membranes.

isolated from the rest of the cerebellar cortex showed that because of their much larger size and distinct morphology Purkinje neurone nuclei could be easily and unequivocally identified in crude homogenates of the cerebellar cortex (Fig. 1*D*). This allowed us to simplify the procedure considerably. In the later stages of the work we picked out Purkinje neurone nuclei directly from homogenates of the cerebellar cortex. The homogenate was placed into a working chamber on the stage of the Leica DMIRB inverted microscope. The nuclei were allowed to attach to the glass bottom of the chamber and then washed from debris with solution (further referred to as KCl solution) containing (mM): KCl 150, Na_2ATP 0.5, K_2EGTA 0.53, CaEGTA 1.47 (calculated free Ca^{2+} concentration 250 nM) and Hepes-KOH 10 (pH 7.3). To obtain access to the inner nuclear membrane the isolated nuclei were treated with sodium citrate as follows. The homogenate was centrifuged for 15 min at 1000 *g*, and the resulting pellet was suspended in homogenization medium with 1% (w/v) sodium citrate added. The suspension was incubated for 30–60 min on ice while stirring gently and then used in experiments as described above.

Electrophysiology

Single ion channels were recorded from nucleus-attached and excised patches of the nuclear membrane in the voltage-clamp mode of the patch-clamp technique. Patch pipettes were prepared from borosilicate glass (Sutter Instrument, Novato, CA, USA) and filled, unless stated otherwise, with KCl solution. Pipette resistances ranged from 7 to 15 MΩ. Data acquisition was carried out using a Visual Patch VP-500 amplifier (Bio-Logic, Claix, France). Currents were filtered with a low-pass Bessel filter at 1 kHz, digitized at 5 kHz and stored on a computer disk. The indifferent electrode was an Ag–AgCl plug connected to a bath chamber via an agar bridge. In all recordings the potential of the bath solution was considered to be 0 mV. The permeability ratio P_a/P_b of ion species *a* and *b* was calculated using the following formula (see, e.g. Miedema, 2002):

$$\frac{P_a}{P_b} = -\frac{z_b^2(b_p - b_b \exp(-z_b E_{rev} F/RT))}{z_a^2(a_p - a_b \exp(-z_a E_{rev} F/RT))} \times \frac{(1 - \exp(-z_a E_{rev} F/RT))}{(1 - \exp(-z_b E_{rev} F/RT))}$$

where a_p, b_p and a_b, b_b refer to the activities of ion species *a* and *b* in, respectively, pipette and bath solution; z_a and z_b are valences of *a* and *b*, E_{rev} is the reversal potential of the single channel current, and *R*, *T* and *F* are standard thermodynamic parameters.

The dependence of $InsP_3$-activated channels on $InsP_3$ concentration was fitted with the Hill equation:

$$\frac{P_o}{P_{max}} = \frac{x^h}{x^h + K^h}$$

where P_o is the open probability of the channel at a given $InsP_3$ concentration *x*, P_{max} is its maximum open probability, *K* is half-maximum activating $InsP_3$ concentration (EC_{50}) and *h* is the Hill coefficient.

Electron microscopy

Nuclei were pelleted at 1000 *g* in homogenization medium. The pellet was fixed for 18 h in 2% glutaraldehyde–PBS and postfixed in 1% osmium tetraoxide–PBS, pH 7.4. Samples were dehydrated in ethanol and propylene oxide and then embedded in epon.

Results

Morphology of isolated neuronal nuclei

Nuclei isolated from Purkinje neurones had a spherical or slightly ellipsoidal shape with an average diameter of $11.8 \pm 1.0\,\mu$m ($n = 34$; Fig. 1*B*). The nuclei had characteristically light nucleoplasm with a clearly visible large dark nucleolus. The nuclei from granule neurones were much smaller with an average diameter of $6.8 \pm 0.7\,\mu$m ($n = 78$; Fig. 1*C*). Because of their large size and distinct morphology, nuclei from Purkinje neurones were quite different from nuclei of any other cerebellar cells and could be easily identified in rough homogenates of the cerebellar cortex (Fig. 1*D*). Transmission electron microscopy showed that the nuclear envelopes of isolated nuclei had a typical double-membrane structure with well-preserved outer and inner nuclear membranes (Fig. 1*E*). The integrity of isolated nuclear envelopes was also confirmed by their staining with fluo-3 AM (see Fig. S1 in Supplemental material). Therefore the outer nuclear membrane was readily accessible for patch-clamp recording in this preparation.

Spontaneously active ion channels in the nuclear membrane of Purkinje neurones

The majority of membrane patches (137 out of 189) from the nuclear membrane of Purkinje neurones contained large conductance ion channels. The channels were characterized by high density (usually 3–5 channels per patch), high open probability and slow kinetics (Fig. 2*A*). In symmetrical KCl solutions the channels had a linear current–voltage relationship (Fig. 2*B*, ●) with a slope conductance of 198 ± 27 pS ($n = 58$).

To determine the selectivity of this channel to K^+ and Cl^- we substituted the isotonic KCl solution in the

bath with hypotonic solutions, which contained reduced concentrations of KCl. In these conditions the equilibrium potential for K^+ was shifted to the left and that for Cl^- to the right. Consequently the current–voltage relationship of an anionic channel would shift to the right and that of a cationic channel to the left. The magnitude of the shift depends on the ratio of the channel permeability to K^+ and Cl^-. The reversal potential of a non-selective channel would not change.

When standard KCl bath solution was substituted with a hypotonic solution (in different experiments the concentration of KCl in the bath solution varied from 10 to 40 mM) the current–voltage relationship of the channel shifted to the left toward the reversal potential for K^+ (Fig. 2*B*, ○). When the pipette solution contained 155 mM K^+ and 150 mM Cl^- and the bath solution contained 15 mM K^+ and 10 mM Cl^- the calculated equilibrium potential for K^+ was −59 mV. In these experimental conditions the reversal potential of the large conductance channel was -48 ± 1 mV ($n = 3$). The calculated ratio of the channel permeabilities for Cl^- and K^+ (P_{Cl}/P_K) was 0.05. Therefore the large-conductance channel in the nuclear membrane of Purkinje neurones was selective to cations.

To study the permeability of the large conductance channel to other monovalent cations, K^+ in the bath solution was replaced with an equimolar amount of the test cation (Fig. 3*A*). The substitution of K^+ in the bath solution with Na^+ shifted the reversal potential of the channels to -10.5 ± 1 mV ($n = 3$) indicating that the channel is permeable to Na^+ with the permeability ratio $P_{Na}/P_K = 0.65$ (Fig. 3*A*, ●). The slope conductance for inward current, carried by Na^+ through the channel, was 94 pS ($n = 3$). The channel was also permeable to Cs^+ (not illustrated). When K^+ in the bath solution was replaced with the large organic monovalent cation *N*-methyl-D-glucamine (NMDG) no inward current

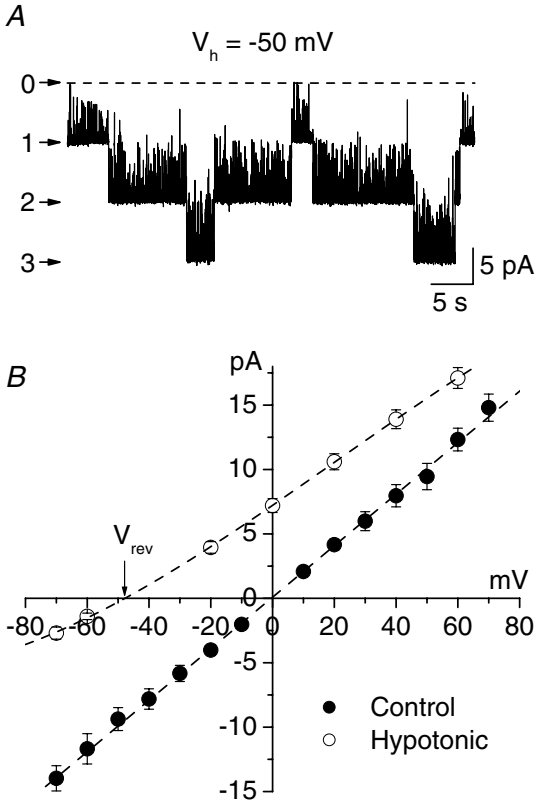

Figure 2. Large conductance channel in the nuclear membrane of Purkinje neurones
A, large conductance channels in an excised patch from the outer nuclear membrane of a Purkinje cell. The patch contained three channels; the zero current level is indicated by a dotted line. Note the slow kinetics and bursting activity of the channel. *B*, current–voltage relationships of the large conductance channel. In symmetrical standard KCl solution the slope conductance of the channel was 198 ± 27 pS ($n = 58$, ●). After substitution of standard KCl bath solution with low (15 mM) KCl solution the reversal potential of the current shifted to −48.6 mV (V_{rev}) suggesting cationic selectivity of the channel (○).

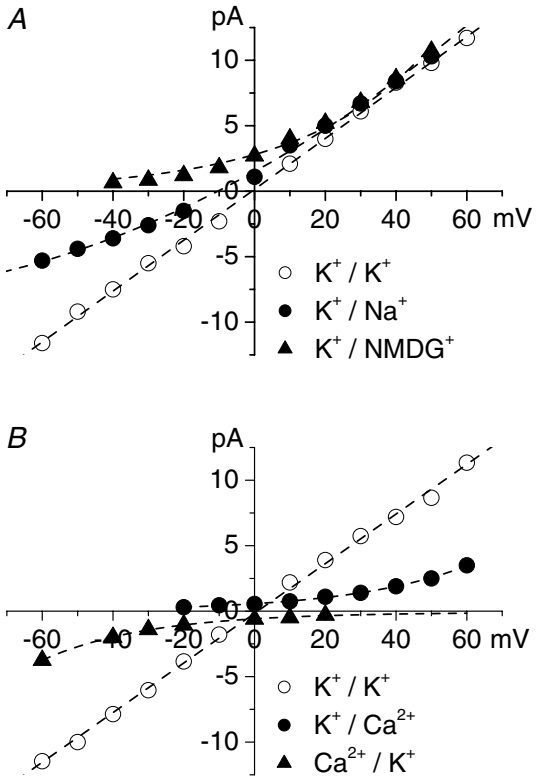

Figure 3. The large conductance channel is a poorly selective K^+ channel
A, permeability of the large conductance channel to monovalent cations. Substitution of KCl in the bath solution with equimolar NaCl (●) shifted the reversal potential of the current through the channel to −10.4 mV suggesting that the channel is permeable to Na^+ with $P_{Na}/P_K = 0.65$. When K^+ in the bath solution was substituted with $NMDG^+$ (▲) no inward current was observed. *B*, when $CaCl_2$ solution in the bath (▲) or in patch pipettes (●) was substituted for KCl solution no Ca^{2+} inward or outward current, respectively, was observed suggesting that the channel is impermeable to Ca^{2+}.

through the channels was recorded (Fig. 3*A*, ▲). Therefore the large conductance channel was a poorly selective K^+ channel.

To study the permeability of the large conductance channels to Ca^{2+} either the working chamber ($n = 6$) or patch pipettes ($n = 4$) were filled with isotonic $CaCl_2$ solution with standard KCl solution at the opposite side of the membrane (Fig. 3*B*). In both cases only K^+ currents through the channels were recorded. In symmetric $CaCl_2$ solution no channel activity was observed in the range from −150 to 150 mV. These data suggest that the large conductance channel is practically impermeable to Ca^{2+}. Similar experiments with $BaCl_2$ solutions suggest that the channels are also impermeable to Ba^{2+} (not illustrated).

The potassium channel blockers tetraethylammonium (10 mM) and 4-aminopyridine (2 mM) had no effect on either the conductance or gating of the large conductance channel. The non-selective blocker of cationic channels La^{3+} at a concentration of 10–100 μM was also ineffective. The ryanodine receptor blocker ruthenium red (10 μM), the ryanodine receptor agonist/blocker ryanodine (1–10 μM), $InsP_3$ (10 μM), and the $InsP_3$ receptor blocker heparin did not affect the large conductance cation channel. Large conductance channels were also unaffected by ATP, Ca^{2+} and Mg^{2+} at physiological concentrations (0.5–5 mM, 0.05–50 μM and 1–5 mM, respectively). At very high concentrations (100 mM) Ca^{2+} and Ba^{2+} on either side of the membrane reduced K^+ currents through the channel (Fig. 3*B*).

The large conductance channels were by far the most abundant type of channel in the nuclear membrane of Purkinje cells. Nevertheless, in a small fraction of patches (11 out of 189 patches) of the nuclear membrane from Purkinje neurones a channel with smaller amplitude and very different pattern of activity was observed (Fig. 4*A*). The relative rarity of the channel and the abundance of the large conductance cationic channels prevented us from studying this channel in detail. The channel activity was characterized by fast fluctuations between multiple subconductance states. The slope conductances of the more frequently occurring subconductance states were 67 ± 5, 52 ± 4 and 27 ± 3 pS ($n = 6$). To determine the ionic selectivity of the channel, KCl in the bath solution was partly replaced with equimolar amounts of potassium gluconate. In these conditions the reversal potential of the channel shifted to positive values close to the equilibrium potential for Cl^- (Fig. 4*B*). These data suggest that the channel is selective for Cl^- with little if any permeability to K^+ and gluconate.

$InsP_3$-activated ion channels in the nuclear membrane of Purkinje neurones

To record $InsP_3$-activated channels the patch pipette or the bath was filled with KCl solution containing 10 μM $InsP_3$. This solution also contained Na_2ATP and free Ca^{2+} in concentrations reported to be optimal for cerebellar $InsP_3R$ stimulation (0.5 mM and 250 nM, respectively; Bezprozvanny *et al.* 1991). Patch-clamp recording from the outer nuclear membrane of Purkinje neurones has revealed the presence of large-conductance spontaneously active ion channels, but $InsP_3$-containing solution did not activate any additional ion channels in excised as well as nucleus-attached nuclear patches ($n \gg 153$).

To access the inner nuclear membrane isolated nuclei were treated with sodium citrate (see Methods). It has previously been reported that citrate selectively removes the outer membrane of the nuclear envelope leaving the inner nuclear membrane intact (Humbert *et al.* 1996).

The application of $InsP_3$ via bath solution and therefore to the nucleoplasmic side of the nuclear membrane of citric acid-treated nuclei activated multiple ion channels in most excised patches (136 out of 153 patches, Fig. 5). The same response was also achieved with nucleus-attached patches. The competitive blocker of $InsP_3Rs$, heparin (0.02–0.4 mg ml^{-1}) added to the nucleoplasmic side of the

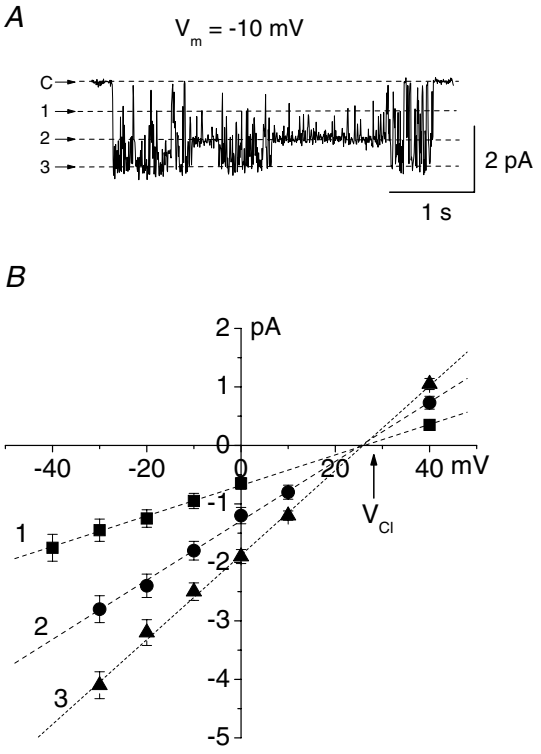

Figure 4. Anion channels in the nuclear membrane of Purkinje neurones
A, the channel demonstrated fast transitions between multiple subconductance states. Three of the subconductance states and the closed state (C) are shown by dashed lines. *B*, current–voltage relationships of the three subconductance states of the anion channel. The arrow indicates the equilibrium potential for Cl^- (V_{Cl}). Pipettes contained standard KCl solution, in bath solution 100 mM KCl was substituted with potassium gluconate.

patch, suppressed the channel activity ($n = 3$). $InsP_3$ in the pipette solution (at the luminal side of the membrane) was ineffective. Therefore the internal nuclear membrane of Purkinje neurones contained $InsP_3Rs$ with their receptor loci directed toward the nucleoplasm. In symmetric KCl solutions the $InsP_3$-activated channels had a linear current–voltage relationship with a slope conductance of 356 ± 4 pS ($n = 7$, Fig. 6*A* and *C*, ●).

$InsP_3$ receptors in the endoplasmic reticulum are Ca^{2+}-selective channels, which are also permeable to other cations such as K^+ and Ba^{2+} (Bezprozvanny & Ehrlich, 1994; Boehning *et al.* 2001). An $InsP_3$-activated Na^+ channel impermeable to Ca^{2+} has also been reported (Somasundaram & Mahaut-Smith, 1995). To determine the permeability of the channel to K^+ and Cl^- we substituted the large organic monovalent cation $NMDG^+$ mole for mole for K^+ in the patch pipette solution. Under these conditions only inward $InsP_3$-activated currents were recorded, suggesting that the $InsP_3$-activated channels were permeable to K^+ with little if any permeability to Cl^- and $NMDG^+$ ($n = 4$, Fig. 6*B* and *C*, ○). To determine the Ca^{2+} permeability of the channel, patch pipettes were filled with solution containing 50 mM $CaCl_2$ and 30 mM KCl. The working chamber contained standard KCl solution with 10 μM $InsP_3$ and 200 nM free Ca^{2+}. Under these conditions the reversal potential of the $InsP_3$-activated channels was -20.7 ± 0.5 mV ($n = 4$; Fig. 6*D* and *F*, ■). The calculated permeability ratio for Ca^{2+} and K^+ was $P_{Ca}/P_K = 5.2$. The $InsP_3$-activated channels were also permeable to Ba^{2+} (Fig. 6*E* and *F*, □). When patch pipettes were filled with solution containing 100 mM $BaCl_2$ and 10 mM Hepes-KOH (pH 7.3) the current through $InsP_3$-activated channels had a reversal potential of -34 ± 0.8 mV ($n = 3$), corresponding to $P_{Ba}/P_K = 5.7$. The single-channel conductance of $InsP_3Rs$ with Ba^{2+} as the current carrier in the potential range from −20 mV to 60 mV was 121 ± 2 pS ($n = 3$). Therefore nuclear $InsP_3Rs$ were Ca^{2+} channels with conductance and selectivity similar to those previously reported for cerebellar $InsP_3Rs$ incorporated into artificial lipid bilayers and recombinant type 1 $InsP_3Rs$ expressed in mammalian cells (Bezprozvanny & Ehrlich, 1994; Boehning *et al.* 2001).

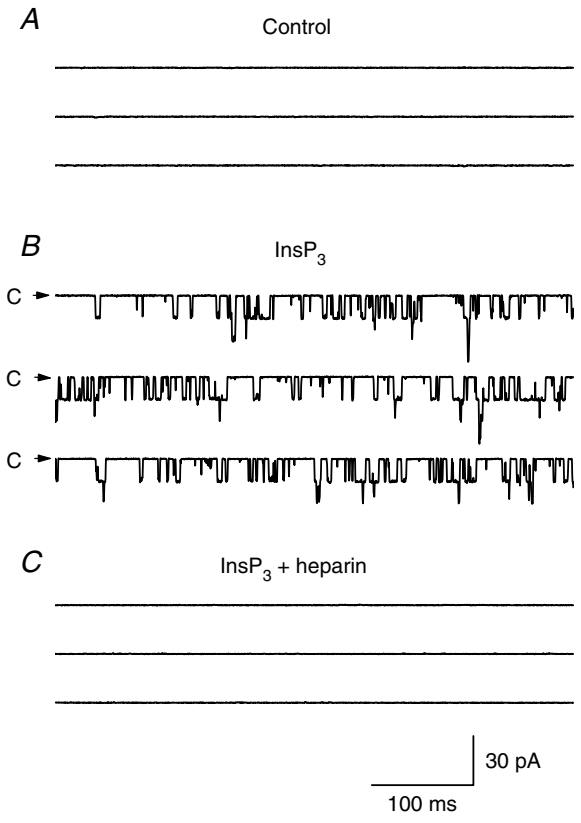

Figure 5. $InsP_3$-activated ion channels in the internal nuclear membrane of Purkinje cells
A, in the absence of $InsP_3$ no channels were recorded. *B*, $InsP_3$ (10 μM) added to bath solution (the nucleoplasmic side of the membrane) activated at least 3 channels, shown as downward deflections from the zero current level indicated by arrows. *C*, the blocker of $InsP_3Rs$, heparin (0.4 mg ml^{-1}), inhibited the channel activity. The bath and pipette contained standard KCl solution with Na_2ATP (0.5 mM) and Ca^{2+} (250 nM). Holding potential was −40 mV.

The open probability of the $InsP_3$-activated channels (P_o) depended on the ion species carrying current through the channel (Fig. S4 of Supplemental material). At a membrane potential of 60 mV in symmetrical KCl solution containing 10 μM $InsP_3$, 250 nM free Ca^{2+} and 0.5 mM ATP, P_o was 0.036 ± 0.04 ($n = 3$). With Ba^{2+} as a current carrier (patch pipettes were filled with 100 mM $BaCl_2$) under the same conditions P_o increased to 0.32 ± 0.03 ($n = 3$). The presence of Ca^{2+} in the pipette solution in concentrations ≥ 10 mM strongly decreased the P_o of outward currents through $InsP_3Rs$ (Fig. 6*D*). When patch pipettes contained 50 mM $CaCl_2$ and 30 mM KCl, the P_o of single outward $InsP_3$-activated currents was reduced to 0.0023 ± 0.0003 ($n = 4$). The decrease in P_o apparently results from the inhibitory effect of high $[Ca^{2+}]$ in the vicinity of the receptor produced by Ca^{2+} current through the channel rather than an effect of the presence of Ca^{2+} at the luminal surface of the membrane, because the P_o of single inward K^+ currents remained practically unchanged (0.038 ± 0.006, $n = 4$, $V_h = -60$ mV.

Most patches of the inner nuclear membrane contained both $InsP_3Rs$ and large conductance cationic channels (see Figs S3 and S4 of Supplemental material). The superposition of multiple large conductance cationic channels complicated our study of $InsP_3Rs$. To separate the two channels in the following experiments Ba^{2+} was used as the current carrier. Patch pipettes were filled with solution containing (mM): $BaCl_2$, 100 and Hepes-KOH, 10 (pH 7.3). $InsP_3$-activated currents were recorded at positive membrane potentials. The bath contained standard KCl solution with $InsP_3$ and free

Ca^{2+} as indicated. As we mention above, 198 pS channels were impermeable to Ba^{2+} and partly inhibited by large concentrations of Ba^{2+}. Thus at positive membrane potentials currents through these channels were negligible.

In the absence of $InsP_3$, Ca^{2+} alone (20 nM–50 μM) was unable to activate the channels. In the presence of 250 nM free Ca^{2+} $InsP_3$ evoked a noticeable activity of the channels in concentrations > 0.1 μM and fully activated $InsP_3$Rs in concentrations ≥ 2–3 μM ($n = 5$, Fig. 7). The experimental data can be fitted by the Hill equation with $EC_{50} = 0.68$ μM and a Hill coefficient of 2.5.

It has previously been reported that Ca^{2+} can both activate and, at higher concentrations, inhibit $InsP_3$Rs (Iino, 1990; Bezprozvanny *et al.* 1991; Finch *et al.* 1991). In *Xenopus* oocytes the inhibitory effect of Ca^{2+} on $InsP_3$Rs was eliminated by treatment with saturating concentrations of $InsP_3$ (Mak *et al.* 1998). Therefore we studied the Ca^{2+} dependence of $InsP_3$Rs at both low (0.3 μM, about 7% of the maximum response; $n = 5$) and saturated (10 μM, $n = 7$) concentrations of $InsP_3$ (Fig. 8 and Fig. S5 of Supplemental material). At both $InsP_3$ concentrations the Ca^{2+} dependence of the $InsP_3$-activated channels was very similar with the maximum activity of the channel observed at $[Ca^{2+}]_i$ in the range from 200 to 400 nM and strong inhibition of $InsP_3$Rs by $[Ca^{2+}]_i \geq 1$ μM.

Apart from $InsP_3$Rs, ryanodine receptors have also been reported in Purkinje neurones (Pozzan *et al.* 1994). Therefore we tried to detect these channels in the nuclear membrane of Purkinje neurones. The agonists of ryanodine receptors, Ca^{2+} (10–50 μM) and cyclic adenosine 5′-diphosphate-ribose (cADPR, 10 μM), activated channels neither in the outer ($n = 56$ and 17, respectively) nor in the inner ($n = 23$ and 12) nuclear membrane of Purkinje neurones. The agonist of putative Ca^{2+}-releasing channels, nicotinic acid adenine dinucleotide phosphate (NAADP, 20–50 nM), was also ineffective ($n = 15$).

Ion channels in the nuclear membrane of granule neurones

In sharp contrast to Purkinje neurones no large-conductance cationic channels were recorded in the nuclear membrane of cerebellar granule neurones. The total density of the channels was also much lower. The most common channel in the nuclear membrane of granule neurones (17 out of 78 patches) had a pattern

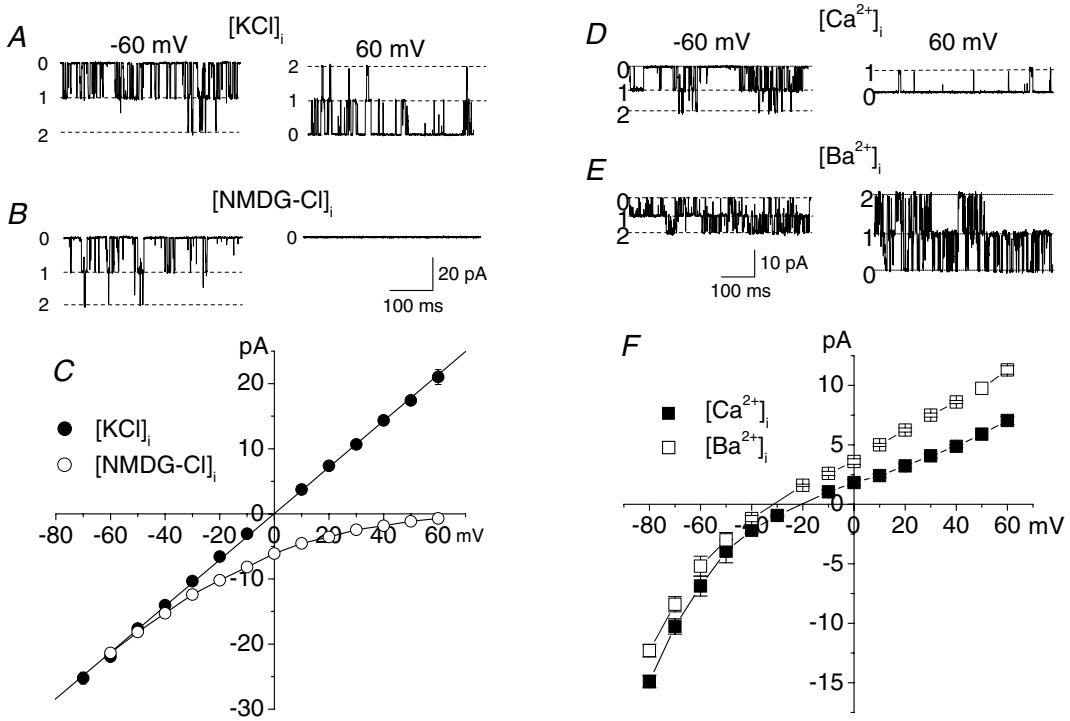

Figure 6. $InsP_3$-activated channels are selective to Ca^{2+}
In symmetrical KCl solution (*A* and *C*, ●) the current–voltage relationship of $InsP_3$-activated channels had a slope conductance of 356 ± 4 pS. After K^+ in the pipette solution was substituted by $NMDG^+$ (*B* and *C*, ○) only inward currents were recorded indicating that the channels were permeable to K^+ and impermeable to Cl^-. Single $InsP_3$-activated currents when patch pipettes were filled with high Ca^{2+} (*D* and *F*, ■) or Ba^{2+} (*E* and *F*, □) solution indicate that the channel was permeable to Ca^{2+} and Ba^{2+} with $P_{Ca}/P_K = 5.2$ and $P_{Ba}/P_K = 5.7$.

of activity very similar to that of the anionic channel in Purkinje neurones (Fig. 9*A* and *B*). The channel quickly fluctuated between multiple subconductance states. The conductances of the more common subconductance states were 46 ± 5 and 18.8 ± 2.1 pS ($n = 4$). When Cl^- in the bath solution was replaced with equimolar amount of gluconate the current–voltage relationship of the channel shifted to the right and was close to the reversal potential for Cl^- (Fig. 9*C*). When 125 mM of the total 150 mM KCl was replaced with potassium gluconate the reversal potential of the current was 40.25 ± 1.3 mV ($n = 4$). These data indicate that the channel is selectively permeable to Cl^- with P_K/P_{Cl} and $P_{Glu}/P_{Cl} < 0.05$.

The nuclear membranes of granule neurones also contained a channel with a different pattern of activity (Fig. 10*A*). It was found in 6 out of 78 patches. In symmetrical KCl solution the channel demonstrated small inward rectification with the slope conductance at negative membrane potentials of 53 ± 4 pS ($n = 5$, Fig. 10*B*). The substitution of isotonic KCl solution with hypotonic solutions with reduced concentration of KCl shifted the reversal potential of the channel to the left suggesting cationic selectivity of the channel (not illustrated).

In experimental conditions identical to those used for recording $InsP_3Rs$ in nuclear membranes of Purkinje neurones, $InsP_3$ (10 μM) in KCl solution containing 0.5 mM ATP and 250 nM free Ca^{2+} failed to activated $InsP_3Rs$ in either the outer ($n = 32$) or the inner ($n = 26$) nuclear membrane of granule neurones.

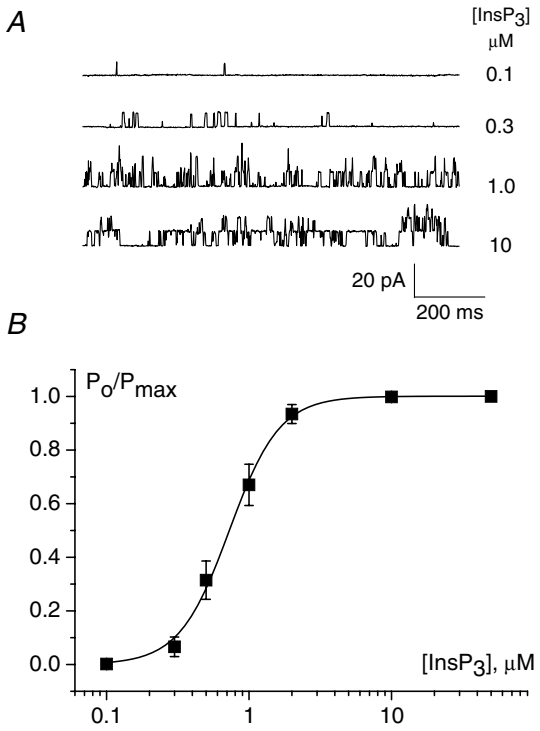

Figure 7. Dependence of nuclear $InsP_3$-activated channels on $InsP_3$ concentration
A, $InsP_3$-activated channel activity at different $InsP_3$ concentrations. *B*, dependence of the normalized open probability of $InsP_3$-activated channels on $InsP_3$ concentration. Data points represent mean ± S.E.M. of five experiments. The continuous curve is the Hill equation fit with $EC_{50} = 0.68$ μM and Hill coefficient = 2.5. Patch pipettes were filled with $BaCl_2$ solution, bath contained standard KCl solution with $[Ca^{2+}]_i = 250$ nM. Holding potential was 40 mV.

Discussion

Here we have presented a method for isolating nuclei from identified neurones of the brain. We have employed this technique to isolate nuclei from cerebellar Purkinje and granule neurones, but the same approach can be readily used for isolating nuclei from any other brain cells (see Fig. S2 of Supplemental material). The significance of studying properties of nuclei from particular types of brain cells is illustrated by the findings of this work.

The major finding is that cerebellar Purkinje and granule cells express different sets of ion channels in their nuclear membranes and therefore their nuclear envelopes may play distinct functional roles. Distinct types of spontaneously active ion channels have also been reported in the outer nuclear membrane of B- and T-lymphocyte cell lines (Franco-Obregon *et al.* 2000). The inner, but not the outer, nuclear membrane of Purkinje neurones contained multiple $InsP_3$-activated channels with their receptor loci facing the nucleoplasm. No $InsP_3Rs$ were found in the nuclear membrane of granule neurones. Although *in vitro* expression of $InsP_3Rs$ in cerebellar granule neurones can be induced artificially (Choi *et al.* 2004), *in vivo* these neurones express little if any $InsP_3Rs$ (Pozzan *et al.* 1994; Taylor *et al.* 1999). Therefore the absence of $InsP_3$-activated channels in the nuclear membranes of granule neurones in our experiments is hardly surprising. On the other hand, Purkinje neurones express the highest level of $InsP_3Rs$ among mammalian cells and their absence in the outer nuclear membrane needs some discussion.

It is well-established that the endoplasmic reticulum is a functionally heterogeneous organelle (reviewed by Meldolesi & Pozzan, 1998; Papp *et al.* 2003). Ca^{2+}-releasing channels as well as other components of Ca^{2+}-signalling machinery are distributed very unevenly throughout the endoplasmic reticulum. In particular in Purkinje neurones $InsP_3Rs$ can be packed in stacks of parallel cisternae at a density about 100 times higher than in the rest of the endoplasmic reticulum and are practically absent in some other regions (Ross *et al.* 1989; Satoh *et al.* 1990). $InsP_3Rs$ in Purkinje neurones were found in perinuclear cisternae although the exact localization of the receptors to the inner or outer nuclear membrane was uncertain.

The localization and orientation of the Ca^{2+}-release channels in the nuclear membrane that we report suggest that the nuclear envelope in Purkinje neurones is

specialized to release Ca^{2+} directly into the nucleoplasm. This is the first evidence for the existence of intranuclear sources of Ca^{2+} in neurones. Ca^{2+} release from the nuclear envelope may amplify Ca^{2+} signals penetrating into the nucleus from the cytoplasm or generate Ca^{2+} transients in the nucleus independently from the cytoplasm. This hypothesis also makes it possible to explain the distribution of $InsP_3Rs$ in the nuclear membrane – $InsP_3Rs$ in the outer nuclear membrane would release Ca^{2+} into the cytoplasm and therefore would reduce Ca^{2+} transients inside the nucleus.

Purkinje neurones predominantly express type 1 $InsP_3Rs$ although small amounts (~1–3%) of type 2 and 3 receptors have also been reported (De Smedt *et al.* 1994; Taylor *et al.* 1999). Nuclear receptors comprise only a small fraction of the total pool of $InsP_3Rs$ in Purkinje neurones and therefore their identity may differ from $InsP_3Rs$ of the rest of the cell. Different types of $InsP_3Rs$ have similar ion selectivity and conductance, but greatly vary in their sensitivity to $InsP_3$ (Thrower *et al.* 2001). The $InsP_3Rs$ studied here had an $EC_{50} = 0.68\ \mu M$ that is close to values (0.194–0.5 μM) reported for cerebellar type 1 $InsP_3Rs$ incorporated into artificial lipid bilayers, but differs from the EC_{50} of type 2 and 3 receptors (58 nM and 3.2 μM, respectively; Ramos-Franco *et al.* 1998; Hagar & Ehrlich, 2000). These data suggest that the $InsP_3Rs$ expressed in the inner nuclear membrane of Purkinje neurones are likely to be type 1.

Our values for EC_{50} (0.68 μM) and the Hill coefficient (2.5) of $InsP_3Rs$ in Purkinje neurone nuclei were somewhat higher than those reported for cerebellar $InsP_3Rs$ incorporated into artificial lipid bilayers (0.194 μM and 0.96 reported by Ramos-Franco *et al.* 1998; 0.5 μM and 1.7 reported by Hagar & Ehrlich, 2000). $InsP_3Rs$ have several putative phosphorylation sites (Thrower *et al.* 2001). It has been reported that phosphorylation by protein kinase A of recombinant type 1 $InsP_3Rs$ expressed in insect cells increased the P_o of the channels incorporated into artificial lipid bilayers more than 10-fold (from < 2–3% to 30–40%) and increased their sensitivity to $InsP_3$ about 4-fold (Tang *et al.* 2003). Different level of phosphorylation may in principle explain differences in EC_{50} reported by different authors. Tang *et al.* (2003) estimated the EC_{50} of phosphorylated $InsP_3Rs$ to be < 50 nM. If the

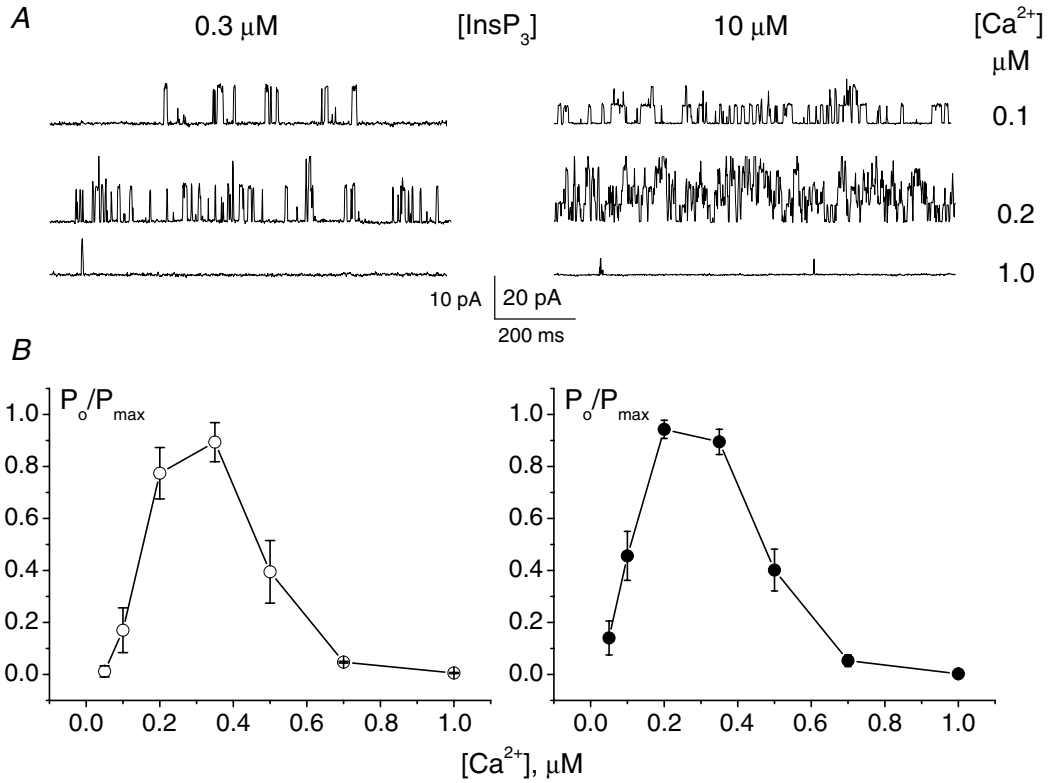

Figure 8. Dependence of $InsP_3$-activated channels on Ca^{2+} concentration at the nucleoplasmic side of the membrane

Channel activity (*A*) and normalized open probability of $InsP_3$-activated channels (*B*) at different Ca^{2+} concentrations in the presence of low (0.3 μM, left; $n = 5$) and saturated (10 μM, right; $n = 7$) $InsP_3$ concentrations. At both $InsP_3$ concentrations 1 μM of Ca^{2+} almost completely inhibited the channel activity. Patch pipettes were filled with $BaCl_2$ solution, bath contained standard KCl solution. Holding potential was 40 mV.

values obtained for heterologously expressed $InsP_3Rs$ hold for native mammalian receptors then because of the high EC_{50} of the nuclear $InsP_3Rs$ they must have been dephosphorylated. On the other hand, the P_o of the nuclear $InsP_3Rs$ with Ba^{2+} as the current carrier was 0.32 which is close to the P_o of phosphorylated recombinant $InsP_3Rs$. Therefore most of the nuclear $InsP_3Rs$ were apparently phosphorylated. These data suggest that the properties of heterologously expressed and native $InsP_3Rs$ may, at least quantitatively, differ. The cause of this discrepancy is unknown and the effects of phosphorylation on native $InsP_3Rs$ need further investigation.

Experiments on permeabilized cells and with flash photolysis of caged $InsP_3$ in cerebellar slices have demonstrated that $InsP_3Rs$ in the cytoplasm of Purkinje neurones are 20–50 times less sensitive to $InsP_3$ than the same receptors incorporated into artificial lipid bilayers (Khodakhah & Ogden, 1993; Fujiwara *et al.* 2001). The reason for this difference is not known and may result from the presence in Purkinje neurones of intrinsic inhibitors of $InsP_3Rs$ (Yang *et al.* 2002; Haynes *et al.* 2004; Kasri *et al.* 2004). In contrast to cytoplasmic receptors, $InsP_3Rs$ in the native nuclear membrane of Purkinje neurones were highly sensitive to $InsP_3$. These data suggest that the nuclear Ca^{2+} store of Purkinje neurones has a much lower threshold for activation by $InsP_3$ than the rest of the endoplasmic reticulum. Similar differences in the sensitivity of nuclear and endoplasmic reticulum $InsP_3Rs$ have been reported in a liver cell line, but in that case it was achieved by expression of different types of $InsP_3Rs$ (Leite *et al.* 2003).

Several groups have reported that mammalian type 1 $InsP_3Rs$ were activated by low (< 300 nM) and inhibited by higher Ca^{2+} concentrations (Iino, 1990; Bezprozvanny *et al.* 1991; Finch *et al.* 1991). In experiments on *Xenopus* oocytes Mak *et al.* (1998) found that an increase in $InsP_3$ concentration about completely eliminated the inhibitory effect of Ca^{2+}. In our experiments Ca^{2+} inhibited $InsP_3Rs$ with the same efficiency both at low (0.3 μM, ~7% of P_{max}) and saturated (10 μM) $InsP_3$ concentrations. Therefore the inhibitory effect of Ca^{2+} on Purkinje neurone nuclear $InsP_3Rs$ did not depend on the $InsP_3$ concentration. Experiments with flash photolysis of caged $InsP_3$ in Purkinje neurones support these data. It has been shown that Ca^{2+} entry through plasmalemmal Ca^{2+} channels strongly suppressed Ca^{2+} release from stores induced by high (25 μM) $InsP_3$ concentrations (Khodakhah & Ogden, 1995). Recombinant type 1 $InsP_3Rs$ expressed in insect

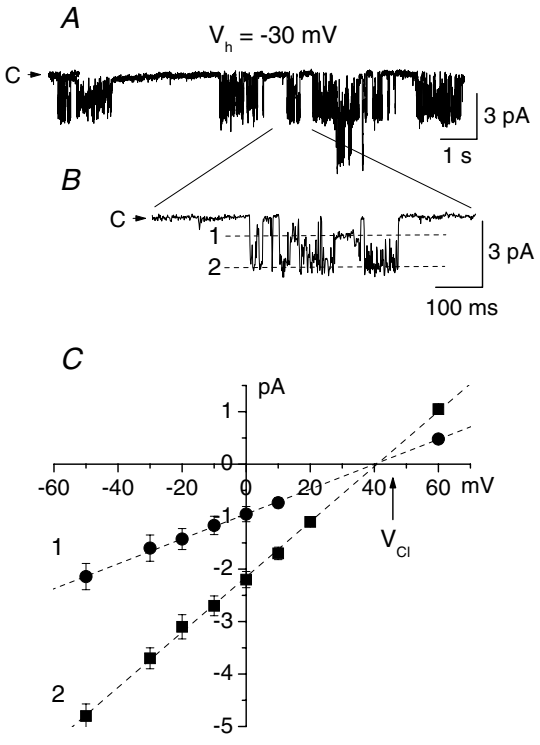

Figure 9. Anion channel in the nuclear membrane of a granule neurone
A and *B*, the anion channel quickly fluctuated between multiple subconductance states. Two of the substates are shown by dashed lines (*B*). *C*, current–voltage relationships of the main conductance substates of the anion channel. The arrow indicates the equilibrium potential for Cl^- (V_{Cl}). Pipettes contained standard KCl solution, in bath solution 125 mM KCl was substituted with potassium gluconate.

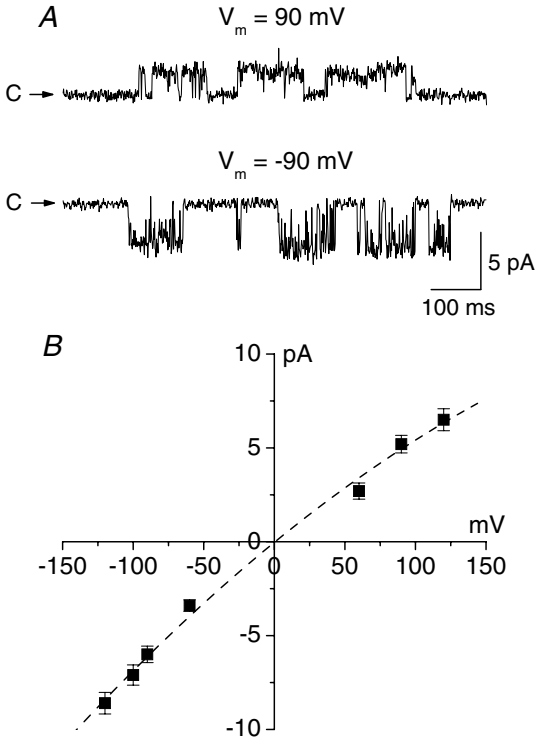

Figure 10. Cation channels in the nuclear membrane of granule cells
A, currents through a single channel at two different holding potentials. *B*, the current–voltage relationship of the cation channel in symmetric KCl solution demonstrated small inward rectification.

cells also demonstrated a bell-shaped Ca^{2+} dependence at saturated $InsP_3$ concentrations and it was not affected by phosphorylation (Tang *et al.* 2003).

There is also other evidence that *Xenopus* $InsP_3Rs$ are not functionally identical to mammalian type 1 receptors. Data presented by Mak *et al.* (1998) suggest that *Xenopus* oocyte $InsP_3Rs$ are much more sensitive to $InsP_3$ than mammalian type 1 receptors. The authors estimated the EC_{50} of *Xenopus* $InsP_3Rs$ to be 50 nM, less by about an order of magnitude than the EC_{50} of mammalian type 1 $InsP_3Rs$. Another difference between the two receptors is the very high open probability of *Xenopus* $InsP_3Rs$ ($P_{max} \sim 0.8$) with long (> 1 s) open bursts, whereas in our experiments in similar ion conditions (K^+ as the current carrier) $P_{max} = 0.034$ and no long-lasting bursts of activity were observed. Studies on type 1 $InsP_3Rs$ incorporated into artificial lipid bilayers also report P_{max} in the range of a few per cent, although these data were obtained in non-physiological ionic conditions (Ramos-Franco *et al.* 1998; Hagar & Ehrlich, 2000).

Xenopus oocyte $InsP_3R$ genes are highly homologous although not identical to mammalian type 1 $InsP_3Rs$ (~93%; Taylor *et al.* 1999). Therefore the discrepancies in properties of $InsP_3Rs$ in *Xenopus* oocytes and Purkinje neurones may result from structural differences between the receptors. An alternative explanation of the differences is suggested by the report that purified cerebellar $InsP_3Rs$ incorporated into artificial lipid bilayers were not inhibited by Ca^{2+} and therefore the inhibition of $InsP_3Rs$ by Ca^{2+} may depend on accessory proteins (Michikawa *et al.* 1999). The assortment of these proteins may be different in *Xenopus* and mammalian cells and this may be the reason for the differences in the properties of $InsP_3Rs$ in these cells.

A decrease in the inhibitory effect of Ca^{2+} was also reported in cerebellar $InsP_3Rs$ incorporated into artificial lipid bilayers, but this effect was observed at unusually high $InsP_3$ concentrations (180 μM) and the physiological relevance of this observation is unclear (Kaftan *et al.* 1997; Thrower *et al.* 2001).

An important effect of saturated $InsP_3$ concentrations was the high activity of $InsP_3Rs$ at resting levels of $[Ca^{2+}]_i$. At $[Ca^{2+}]_i = 100$ nM $InsP_3R$ activity reached about 46% of P_{max} at 10 μM $InsP_3$, compared with about 17% of P_{max} at 0.3 μM $InsP_3$. Taking into account that P_{max} at 0.3 μM $InsP_3$ comprised only 7% of that at 10 μM $[InsP_3]$ the absolute activity of $InsP_3Rs$ at resting $[Ca^{2+}]_i$ rose about 40 times with the rise in $InsP_3$ concentration. The bell-shaped Ca^{2+} dependence of $InsP_3Rs$ is considered to be the cause of Ca^{2+} oscillations seen in many cells (Bezprozvanny *et al.* 1991; Thrower *et al.* 2001). Ca^{2+} oscillations are usually observed at low levels of agonist stimulation whereas stronger stimuli evoke a biphasic Ca^{2+} response (a Ca^{2+} spike followed by a smaller steady-state rise in $[Ca^{2+}]_i$). This effect was explained by Mak *et al.* (1998) by the so called 'tuning' of the receptor, i.e. the elimination of the inhibitory effect of Ca^{2+} on $InsP_3Rs$ at saturated $InsP_3$ concentrations generated by strong stimuli. Our data suggest an alternative explanation of this phenomenon – at large $InsP_3$ concentrations high activity of $InsP_3Rs$ even at low $[Ca^{2+}]_i$ makes the sequestration of Ca^{2+} in the endoplasmic reticulum ineffective and produces biphasic rather than oscillatory responses to agonists.

Yang *et al.* (2002) reported that a family of neuronal Ca^{2+}-binding proteins (CaBP) interacts with $InsP_3$-binding region of $InsP_3Rs$ and showed that a member of this CaBP1 family is an agonist of $InsP_3Rs$ of the outer nuclear membrane of *Xenopus* oocytes. They also demonstrated that CaBP1 is highly expressed and co-localized with $InsP_3Rs$ in Purkinje neurones. The authors proposed that CaBP1 is a protein agonist of $InsP_3Rs$ that enables the channel to be involved in Ca^{2+}-induced Ca^{2+} release in the absence of $InsP_3$ in a manner similar to ryanodine receptors. In our experiments Ca^{2+} alone, in the absence of $InsP_3$, was unable to activate $InsP_3Rs$. CaBP1 is a membrane-localized protein and it is unlikely that it was washed-out during nucleus isolation. These data suggest that CaBP1 is not involved in regulation of nuclear $InsP_3Rs$ in Purkinje neurones. There are several other reports that indicate that $InsP_3$-independent activation of $InsP_3Rs$ is not a universal property of cerebellar $InsP_3Rs$. A local rise in $InsP_3$ in Purkinje neurone dendrites due to synaptic activation of metabotropic glutamate receptors or flash photolysis of caged $InsP_3$ evokes an increase in $[Ca^{2+}]_i$ restricted to the area of stimulation without a significant regenerative propagation of the Ca^{2+} signal, in spite of the reported presence of high levels of CaBP1 throughout the dendritic tree (Finch & Augustine, 1998; Takechi *et al.* 1998). In experiments on other mammalian cells CaBP1 inhibited agonist-evoked $InsP_3$-mediated Ca^{2+} signalling and no rise in $InsP_3$-independent Ca^{2+}-induced Ca^{2+} release was reported by the authors (Haynes *et al.* 2004; Kasri *et al.* 2004). Therefore we, and other authors also, have not obtained any data confirming the agonist action of CaBP1, and its role in $InsP_3R$ regulation remains contentious.

Nuclear envelopes from Purkinje neurones also contained numerous spontaneously active large-conductance channels. The channels had high open probability and were permeable to K^+ and some other monovalent cations, but impermeable to Ca^{2+}. Large-conductance cationic channels resembling the nuclear channels of Purkinje neurones have previously been reported in the sarcoplasmic reticulum of different cells (see, e.g. Picard *et al.* 2002). High permeability to K^+ is generally characteristic of the membrane of endo(sarco)plasmic reticulum Ca^{2+} stores. These channels are thought to provide a route for counterflow

of K^+ to prevent changes in the membrane potential that may arise due to movements of Ca^{2+} through the endoplasmic reticulum membrane. The large-conductance cationic channels in the nuclear membrane of Purkinje neurones may have the same function. This assumption is consistent with the fact that the channels are expressed in $InsP_3$-sensitive nuclear envelopes of Purkinje neurones, but not in the nuclear envelopes of granule neurones.

As we have already mentioned above, $InsP_3$-activated Ca^{2+} channels have been recorded in the outer nuclear membrane of *Xenopus* oocytes (Stehno-Bittel *et al.* 1995; Mak *et al.* 1998). No native $InsP_3$-activated channels have until now been recorded in the nuclear membrane of mammalian cells (Mazzanti *et al.* 2001). On the other hand fluorescent Ca^{2+} measurements indicate that the nuclear envelope of liver and a number of other mammalian cells are $InsP_3$-sensitive Ca^{2+} stores (Nicotera *et al.* 1990; Gerasimenko *et al.* 1995). Incorporation of the nuclear membranes from a hepatocyte cell line into artificial lipid bilayers also revealed the presence of $InsP_3$Rs (Leite *et al.* 2003). The distribution of $InsP_3$Rs in mammalian nuclei has not been unequivocally established. $InsP_3$ binding sites were found in the internal nuclear membrane of hepatocytes (Humbert *et al.* 1996). It has recently been reported that $InsP_3$ can release Ca^{2+} from protrusions of inner nuclear membrane in the nucleoplasm of a hepatocellular carcinoma cell line, indicating the presence of functional $InsP_3$Rs inside the nucleus (Echevarría *et al.* 2003). On the other hand, careful analysis of Ca^{2+} signalling in HeLa cells showed no sources of Ca^{2+} inside the nucleus (Lipp *et al.* 1997).

These discrepancies may, at least partially, be explained by different mechanisms of Ca^{2+} regulation in the nuclei of different cells. In our experiments we were unable to record Ca^{2+} channels in the nuclear membrane of cerebellar granule neurones. Therefore different types of neurones may have distinct mechanisms of regulating Ca^{2+} in the nucleoplasm. The reason for these differences between neurones is unknown. One possibility is that Ca^{2+} channels in the internal nuclear membrane are expressed only in neurones where nuclear Ca^{2+} signalling plays an important role and larger Ca^{2+} transients may be required to trigger downstream events. Alternatively, the existence of a source of Ca^{2+} inside the nucleus of Purkinje neurones may result from the large size of these nuclei. The nucleus of Purkinje neurones is about twice as large in diameter and 8 times larger in volume than the nucleus of granule neurones. The inflow from the cytoplasm may by itself be insufficient to fill this volume with the necessary amount of Ca^{2+}.

$InsP_3$ for modulating the Ca^{2+} excitability of $InsP_3$Rs in the inner nuclear membrane may come from two sources. It may be synthesized in the plasma membrane and enter the nucleus from the cytoplasm through nuclear pores. In neurones this possibility is limited by the fact that only a small fraction of $InsP_3$ synthesized in neurones can reach the nucleus, because the vast majority of the $InsP_3$-synthesizing metabotropic receptors are located in dendrites and produce only a local rise in $InsP_3$ concentration (Finch & Augustine, 1998; Takechi *et al.* 1998). The alternative possibility is that $InsP_3$ may be synthesized inside the nucleus. It has been reported that the nucleus contains a complete set of enzymes of $InsP_3$ metabolism (D'Santos *et al.* 1998; Irvine, 2000). The regulation of these enzymes is poorly understood. In Swiss 3T3 cells stimulation of insulin-like growth factor I (IGF-I) receptors activates inositide metabolism in the nucleus. IGF-I is present in climbing fibres, is released by their electrical stimulation and Purkinje neurones express IGF-I receptors (Ito, 2001). Therefore $InsP_3$ levels in the nucleus of Purkinje neurones may be regulated by IGF-I and/or other neuromodulators independently from the cytoplasm, switching on and off the nuclear envelope Ca^{2+} store.

The physiological role of nuclear Ca^{2+} signalling has not been unequivocally established and may be different in different cells. As we have already mentioned in the introduction, nuclei contain Ca^{2+}-sensitive transcription factors and therefore a rise in nuclear Ca^{2+} can affect gene transcription. It has been hypothesized that nuclear Ca^{2+} may be involved in the formation of transcription-dependent neuronal plasticity (Bading, 2000). A rise in nuclear Ca^{2+} controls CREB-mediated gene expression triggered by synaptic activity (Hardingham *et al.* 2001) . In Purkinje neurones associated stimulation of synaptic inputs from climbing and parallel fibres evokes long-term depression (LTD) of synaptic transmission at excitatory parallel fibre synapses (reviewed by Ito, 2001). LTD induction is Ca^{2+} dependent. Mice with a disrupted type 1 $InsP_3$R gene completely lack LTD suggesting an important role of $InsP_3$Rs in LTD formation (Inoue *et al.* 1998). It has been shown that the late phase of cerebellar LTD requires protein synthesis (Linden, 1996). Inhibition of CREB or CaMKIV function eliminated or strongly suppressed the late phase of LTD (Ahn *et al.* 1999; Ho *et al.* 2000). Therefore the formation of the late phase of LTD depends on Ca^{2+}-sensitive transcription factors. $InsP_3$Rs in the internal nuclear membrane of Purkinje neurones may be involved in regulation of nuclear Ca^{2+} metabolism and induction of late phase of LTD.

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