L-Type Calcium Channels Mediate Calcium Oscillations in Early Postnatal Purkinje Neurons

Patricia Liljelund, Jeffrey G. Netzeband, and Donna L. Gruol

Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037

Ca^{2+} signaling is important in many fundamental neuronal processes including neurotransmission, synaptic plasticity, neuronal development, and gene expression. In cerebellar Purkinje neurons, Ca^{2+} signaling has been studied primarily in the dendritic region where increases in local Ca^{2+} have been shown to occur with both synaptic events and spontaneous electrical activity involving P-type voltage-gated Ca^{2+} channels (VGCCs), the predominant VGCC expressed by Purkinje neurons. Here we show that Ca^{2+} signaling is also a prominent feature of immature Purkinje neurons at developmental stages that precede expression of dendritic structure and involves L-type rather than P-type VGCCs. Immature Purkinje neurons acutely dissociated from postnatal day 4–7 rat pups exhibit spontaneous cytoplasmic Ca^{2+} oscillations. The Ca^{2+} oscillations require entry of extracellular Ca^{2+} , are blocked by tetrodotoxin, are communicated to the nucleus, and correlate closely with patterns of endogenously

generated spontaneous and evoked electrical activity recorded in the neurons. Immunocytochemistry showed that L-, N-, and P/Q-types of VGCCs are present on the somata of the Purkinje neurons at this age. However, only the L-type VGCC antagonist nimodipine effectively antagonized the Ca^{2+} oscillations; inhibitors of P/Q and N-type VGCCs were relatively ineffective. Release of Ca^{2+} from intracellular Ca^{2+} stores significantly amplified the Ca^{2+} signals of external origin. These results show that a somatic signaling pathway that generates intracellular Ca^{2+} oscillations and involves L-type VGCCs and intracellular Ca^{2+} stores plays a prominent role in the Ca^{2+} dynamics of early developing Purkinje neurons and may play an important role in communicating developmental cues to the nucleus.

Key words: cerebellum; development; acutely isolated neurons; Ca^{2+} signaling; nuclear Ca^{2+} ; intracellular Ca^{2+} stores

Cerebellar Purkinje neurons are a favored model for studying Ca^{2+} signaling because of the prominent role played by Ca^{2+} in dendritic excitability and synaptic transmission in this neuronal type (Tank et al., 1988; Hockberger et al., 1989; Gruol et al., 1992; Miyakawa et al., 1992; Eilers et al., 1996). In mature Purkinje neurons, dendritic voltage-gated Ca^{2+} channels (VGCCs), predominantly the P-type (Hillman et al., 1991), have been shown to mediate changes in dendritic Ca^{2+} levels associated with spontaneous or evoked electrical activity (Tank et al., 1988; Sugimori and Llinas, 1990). In addition, stimulation of either climbing fibers or parallel fibers, the excitatory afferents to Purkinje neurons, produces local increases in dendritic Ca^{2+} via activation of glutamate receptors and VGCCs (Ross and Werman, 1987; Sugimori and Llinas, 1990; Konnerth et al., 1992; Miyakawa et al., 1992; Eilers et al., 1995). Release of Ca^{2+} from intracellular Ca^{2+} stores also contributes to Ca^{2+} signaling in Purkinje neuron dendrites and is mediated both by ryanodine receptors (RyR) and inositol-1,4,5-trisphosphate receptors (IP3R) (Kano et al., 1995a; Gruol et al., 1996; Finch and Augustine, 1998; Narasimhan et al., 1998).

Ca^{2+} signaling is also an important physiological property of developing neurons, but relatively little is known about this process in immature Purkinje neurons. Components essential for Ca^{2+} signaling in Purkinje neuron dendrites are expressed early in Purkinje neuron development, when Purkinje neurons are structurally relatively simple and consist primarily of a somata and axon with little or no dendritic structure. These Ca^{2+} -signaling components included VGCCs, RyRs, IP3Rs, and the Ca^{2+} -binding proteins

calbindin and parvalbumin (Gruol and Franklin, 1987; Regan, 1991; Gruol et al., 1992; Mintz et al., 1992; Llano et al., 1994; Milosevic and Zecevic, 1998). Large Ca^{2+} transients can be experimentally evoked in early postnatal Purkinje neurons (Kano et al., 1995b), indicating the functional capacity for Ca^{2+} signaling. However, the relationship between this capacity and the physiology of the immature Purkinje neurons is unknown. Physiological events associated with dendritic Ca^{2+} signaling in mature Purkinje neurons are expressed at the somatic level in early developing Purkinje neurons including endogenously generated electrical activity and excitatory synaptic input (Woodward et al., 1969a,b; Gruol and Franklin, 1987; Mason et al., 1990; Gruol et al., 1991; Altman and Bayer, 1997), but the potential role of this activity in Ca^{2+} signaling has not been investigated.

In the current study we investigated Ca^{2+} dynamics in early developing Purkinje neurons acutely isolated from the cerebella of postnatal rats. Results show that immature Purkinje neurons exhibit spontaneous somatic Ca^{2+} oscillations that are generated by endogenous electrical activity, amplified via release of Ca^{2+} from intracellular stores, and transmitted to the nucleus. The Ca^{2+} oscillations occur via a signaling pathway involving L-type VGCCs rather than P-type VGCCs, which mediate the electrically evoked Ca^{2+} signals of Purkinje neuron dendrites (Usowicz et al., 1992). In addition to playing an important role in regulating Ca^{2+} dynamics of immature Purkinje neurons, this signaling pathway may contribute to the early developmental program by influencing downstream events such as gene expression.

MATERIALS AND METHODS

Preparation of acutely dissociated cerebellar cells. Purkinje neurons were acutely isolated from postnatal rats according to the methods of Surmeier et al. (1991). Rat pups between the ages of postnatal day 4 (P4) and P7 were rapidly decapitated, and the cerebellum was immediately removed and kept moistened with ice-cold, oxygenated artificial CSF (in mM: 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4 , 24 NaHCO_3 , 10 glucose, 0.2 CaCl_2 , and 5.0 MgSO_4). The meninges were dissected away, 350–450 μm sagittal slices were cut manually from the vermis, and the white matter was removed under a dissecting microscope. Slices were minced in isethionate buffer (in mM: 140 sodium isethionate, 2 KCl, 4 MgCl_2 , 23 glucose, and 15 HEPES, pH adjusted to 7.4 with NaOH) supplemented with 5 μM glutathione, 1 mM

Received Jan. 11, 2000; revised July 10, 2000; accepted July 19, 2000.

This work was supported by National Institute on Alcohol Abuse and Alcoholism Training Grant AA07456-17, RO1 AA06665, and RO1 AA06420 to the Alcohol Center. We thank Gilles Martin for generously sharing his protocol for acute isolation of neurons, Lely Quina for her assistance in adapting the method for Purkinje neurons, and Jaimes Schneeloch for assistance with electrophysiological studies. We are also grateful to Floriska Chizer-Slack for secretarial help and Novartis for the gift of CGP55845A.

Correspondence should be addressed to Dr. Donna L. Gruol, Department of Neuropharmacology, CVN 11, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail: gruol@scripps.edu.

Copyright © 2000 Society for Neuroscience 0270-6474/00/207394-10\$15.00/0

N-ω-nitro-L-arginine, and 10 mM kynurenic acid at room temperature (21–23°C). The tissue was incubated 45 min in Earl's Balanced Salt Solution (supplemented as above) in a spinner flask in the presence of 5% CO₂ and 95% O₂ at room temperature. The tissue was then incubated for 30 min with 0.1% papain (Sigma, St. Louis, MO) in HBSS (supplemented as above) in an oxygenated spinner flask maintained at 37°C. The tissue was washed three times in isethionate buffer (see composition above) and gently triturated through a size series of three fire-polished Pasteur pipettes to dissociate the tissue and isolate the cells. Cells were plated on Lab-Tek chamber slides (Nunc, Naperville, IL) for immunological studies or glass coverslips (Fisher Scientific, Houston, TX) for physiological recordings. Experiments were performed in physiological saline (in mM: 140 NaCl, 3.5 KCl, 0.4 KH₂PO₄, 1.25 Na₂HPO₄, 2.2 CaCl₂, 2 MgSO₄, 10 glucose, and 10 HEPES, pH-adjusted to 7.3 with NaOH) or Mg²⁺-free saline supplemented with 5 μM glycine depending on the experiment.

Measurement of Ca²⁺ levels. Dissociated cells were centrifuged to pellet them (5 min at 6000 rpm) and loaded with the fluorescent Ca²⁺ indicator fura-2 AM (Molecular Probes, Eugene, OR) by resuspending the cells in physiological saline (see composition above) containing 3 μM fura-2 and 0.02% pluronic F-127 (Molecular Probes) for 30 min in the dark at room temperature (21–23°C). Cells were then centrifuged and resuspended in physiological saline. The cell suspension was plated on glass coverslips that had been precoated overnight at 4°C with Thy1 antibody (100 μg/ml; Sigma), washed once with PBS, and incubated in physiological saline during the attachment period. Poly-L-lysine (Sigma) and Matrigel (Collaborative Biomedical Products, Bedford, MA) were also tested as substrates, but Thy1 antibody-coated glass coverslips were found to most effectively immobilize the Purkinje neurons (Baptista et al., 1994). Results were not dependent on the relative plating density or substrate used. Coverslips with fura-2-loaded cells were used for experiments 1 hr after plating.

Standard microscopic fura-2 digital imaging techniques were used as described previously (Gruol et al., 1996). Neurons were viewed with a Nikon Eclipse TE300 inverted fluorescent microscope and light intensity monitored with a silicon-intensifier target camera (SITCAM) (MTI VE1000SIT; Dage, Michigan City, IN) for capturing live video images. Data were collected at 2–5 sec intervals (four frames per wavelength were averaged for each time point) over a total time of 90 sec to 10 min depending on the experiment. Ratio images of individual neurons in each field were formed by pixel-by-pixel division of the averaged images col-

lected at 340 and 380 nm (340/380 nm). Real-time digitized display, image acquisition, and calculation of intracellular Ca²⁺ levels were made with Microcomputer Imaging Device imaging software (Imaging Research, St. Catharines, Ontario). Intracellular Ca²⁺ levels were estimated using the following formula: $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) * F_0/F_s$, where R is the ratio value, R_{min} is the ratio for a Ca²⁺-free solution, R_{max} is the ratio for a saturated Ca²⁺ solution, K_d is 135 (the dissociation constant for fura-2), F_0 is the intensity of a Ca²⁺-free solution at 380 nm, and F_s is the intensity of a saturated Ca²⁺ solution at 380 nm. R_{min} and R_{max} values were determined from standardized solutions of known Ca²⁺ concentration (Molecular Probes calibration kit C-3009) containing fura salt (100 μM). Calculated levels of intracellular Ca²⁺ obtained by this method are considered approximations and are used primarily to provide information about the relative level of intracellular Ca²⁺ under a variety of experimental conditions. Typical R_{max} , R_{min} , and F_0/F_s values were 0.61, 2.85, and 2.5, respectively. The low level of background fluorescence and adjustment of the black level of the SITCAM eliminated the need for background subtraction methods. Measurements were made of baseline Ca²⁺ levels, the maximum amplitude of the Ca²⁺ oscillations relative to baseline, and the average Ca²⁺ level above baseline (see Fig. 2B). Baseline for a neuron was defined as the lowest Ca²⁺ level observed in the Ca²⁺ recording.

Drugs. Experiments were performed in the presence of 5 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (Tocris Cookson, Langford, Bristol, UK), 100 μM picrotoxin (Sigma), and 1 μM CGP55845A (Novartis, Basel, Switzerland) in the bath saline to block AMPA, GABA_A, and GABA_B receptors, respectively. Test agents were diluted in bath saline and introduced by bath addition or bath exchange to achieve the desired concentrations. A variety of drugs were tested including the P-type VGCC antagonist ω-agatoxin IVA (0.1 μM; Calbiochem, La Jolla, CA), the N-type VGCC antagonist ω-conotoxin GVIA (1 μM; Calbiochem), the L-type VGCC antagonist nimodipine (1 μM; Research Biochemicals International, Natick, MA), the competitive NMDA receptor antagonist APV (50 μM; Tocris), the NMDA receptor open channel blocker 5R,10S-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine/dizocilpine (MK801; 10 μM; Tocris), the sodium channel inhibitor tetrodotoxin (TTX; 0.5 μM; Calbiochem), and the dihydropyridine receptor agonist S(-)-BayK-8644 (1 μM; Research Biochemicals International). Stock solutions of nimodipine and BayK-8644 were prepared in DMSO.

Figure 1. Morphological and immunological characteristics of acutely isolated immature Purkinje neurons. *A–C*, Live Purkinje neurons photographed under high power (Nomarski optics) to illustrate features of early developing Purkinje neurons at P4 (*A*) to P7 (*B*, *C*). *A*, Perisomatic processes (arrow) were characteristic of Purkinje neurons early in development (P4). The asterisk indicates granule neurons that were present in all microscopic fields. *B*, A short axon (arrow) and prominent lateralized nucleus (arrowhead) were generally evident at P7. *C*, The initial outgrowth of the apical dendritic tree (arrow) was occasionally seen at P7. It should be noted, however, that a variety of morphological forms could be found within any given preparation because the cerebellar folia mature at different rates. *D*, Glial cell (arrowhead) immunostained with an antibody to GFAP. Arrow points to an unstained Purkinje neuron. As noted for acutely prepared P7 to P14 cerebellum (Hockberger et al., 1994), we found that some glial cells resembled Purkinje neurons, especially at the earliest stage studied. However, preliminary removal of the white matter during the dissection significantly reduced the number of glial cells. *E–G*, Purkinje neurons immunostained with an antibody to calbindin, a specific marker for Purkinje neurons (Enderlin et al., 1987). Arrows in *E–G* correspond to the structures labeled in *A–C*, respectively. Granule neurons (*E*, asterisk) did not immunostain for calbindin. *H*, Purkinje neuron (arrow) immunostained with an antibody to GABA, the neurotransmitter used by Purkinje neurons. Granule neurons, which use glutamate as a neurotransmitter, were unstained. All immunostaining was photographed using Hoffman optics. Scale bars: *C*, *H*, 15 μm (refer to *A–C* and *D–H*, respectively).

DMSO by itself did not affect spontaneous Ca^{2+} oscillations (data not shown). In most studies at least three different cell preparations were tested to determine the effect of a given drug. n is the number of cells studied. Results are presented as mean $\pm$ SEM.

Electrophysiological techniques. Electrophysiological recordings were performed in combination with recordings of intracellular Ca^{2+} or in parallel with Ca^{2+} -imaging studies (see above). Current-clamp recordings were made from the somatic region of acutely isolated Purkinje neurons using the nystatin, perforated-patch method of whole-cell recording and the Axopatch-1C amplifier (Axon Instruments, Foster City, CA) following previously described methods (Netzeband et al., 1997). Briefly, patch pipettes (2–4 M Ω) were filled with a pipette solution containing 6 mM NaCl, 154 mM K^{+} -gluconate, 10 mM HEPES, and 200 $\mu\text{g}/\text{ml}$ nystatin (Sigma), pH adjusted to 7.3 with KOH. A stock solution of nystatin (50 mg/ml DMSO) was prepared fresh for each experiment and diluted in the pipette solution. Recordings were made at the resting membrane potential of the cell and monitored on a polygraph (Gould, West Warwick, RI) and oscilloscope. For better resolution of fast events, selected data were recorded on FM tape (Store 4DS; Rascal Recorders, Irvine, CA) for playback at reduced tape speed onto the polygraph recorder. Voltage responses elicited by a standardized series of hyperpolarizing and depolarizing current pulses (500 msec duration) were used to assess membrane properties. pClamp software and the Labmaster interface (Axon Instruments) were used for acquisition and analysis of the current-evoked membrane responses. All recordings were made at room temperature.

Immunohistochemistry. The acutely dissociated cerebellar cells were immunostained according to standard methods (Netzeband et al., 1997). Cells were fixed for 15 min in 4% paraformaldehyde (or 4% paraformaldehyde and 0.1% glutaraldehyde for GABA immunostaining) and washed three times (10 min) with 5% sucrose in PBS and once with PBS (10 min). The cells were then incubated 30 min in PBS containing 0.05% Triton X-100, followed by three washes (10 min) in PBS. Primary antibodies were diluted in PBS with 0.5 mg BSA/ml and 1% serum and incubated overnight at 4°C. The following antibodies were used: mouse monoclonal anti-calbindin (1:3000; Sigma); mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:2000; Boehringer Mannheim, Indianapolis, IN); rabbit polyclonal anti-GABA (1:500; Incstar, Stillwater, MN); rabbit polyclonal anti- α_{1A} subunit of P/Q-type VGCC (1:1000; Alomone Labs, Jerusalem, Israel); rabbit polyclonal anti- α_{1B} subunit of N-type VGCC (1:500; Alomone Labs); and rabbit polyclonal anti-rat α_{1C} subunit of L-type VGCC (1:1000; Alomone Labs). Vectastain kits (Vector Laboratories, Burlingame, CA) were used for the secondary antibodies and the peroxidase reaction.

RESULTS

Developing Purkinje neurons show spontaneous Ca^{2+} oscillations

The experiments focused on the postnatal age interval between P4 and P7 (Fig. 1*A–C*), known to represent a small but significant time window of Purkinje neuron development that precedes the main period of dendritic elaboration and synapse formation (Altman, 1972). Purkinje neurons in cerebellar cell preparations obtained by acute dissociation of P4 to P7 rat cerebella were identified for study by morphological criteria established by immunostaining cerebellar cell preparations with an antibody to the Ca^{2+} -binding protein calbindin (Fig. 1*E–G*), a marker for Purkinje neurons (Enderlin et al., 1987). Morphologically identified Purkinje neurons also immunostained with an antibody to GABA (Fig. 1*H*), the neurotransmitter used by Purkinje neurons, but did not immunostain with an antibody to GFAP (Fig. 1*D*), a marker for glial cells. Granule neurons, cerebellar interneurons that use glutamate as a transmitter, were identified by their small, uniform size and lack of immunoreactivity for antibodies to calbindin, GABA, and GFAP (Fig. 1).

Measurement of intracellular Ca^{2+} levels in the immature Purkinje neurons revealed that they exhibit sustained spontaneous oscillations of intracellular Ca^{2+} (Fig. 2) and that such activity is present as early as 4 d after birth, the earliest age examined. To obtain an overview of the patterns of spontaneous Ca^{2+} activity, collections of 5 or 10 min at 5 sec intervals were performed on oscillating Purkinje neurons isolated from P4 to P7 rat pups. Patterns of Ca^{2+} oscillation were fairly consistent for a given Purkinje neuron but were highly variable among Purkinje neurons at all ages studied. Figure 2 illustrates three typical patterns of Ca^{2+} oscillations observed in the Purkinje neurons. The average frequency of Ca^{2+} oscillations was measured as the number of peaks occurring during the recording period. The average frequency for a population of P4 to P7 neurons studied was 2.6 ± 0.1 oscillations/min ($n = 24$), a rate significantly higher than, for

Figure 2. Early postnatal Purkinje neurons exhibit spontaneous oscillations of intracellular Ca^{2+} levels. *A*, Ca^{2+} recordings from three representative Purkinje neurons; spontaneous Ca^{2+} oscillations persisted throughout the entire recording period. A granule neuron is included in each example for comparison; Ca^{2+} oscillations were small or absent in granule neurons (see also Figs. 4, 6, and 7). Collections were performed at 5 sec intervals for 10 min. The animal age is indicated in the bottom right-hand corner. *B*, Quantification of Ca^{2+} oscillations. Measurements were made using Axograph software (Axon Instruments). The baseline Ca^{2+} level was defined as the lowest Ca^{2+} level measured in a neuron. The highest Ca^{2+} level relative to baseline was defined as the maximum amplitude. Average amplitude was measured as the average Ca^{2+} level above baseline calculated over the recording period.

Table 1. Descriptive characteristics of spontaneous Ca^{2+} transients observed in Purkinje neurons

Age	Non-oscillating cells		Oscillating cells			
	<i>n</i>	Baseline Ca^{2+} (nM)	<i>n</i>	Baseline Ca^{2+} (nM)	Maximum amplitude (nM)	Average Ca^{2+} (nM)
P4	21	54 ± 6	82	48 ± 3	85 ± 8	29 ± 3
P5	33	61 ± 2	79	$62 \pm 2^*$	$47 \pm 5^*$	$16 \pm 2^*$
P6	50	54 ± 3	95	$61 \pm 3^*$	$49 \pm 6^*$	$20 \pm 2^*$
P7	28	61 ± 3	94	$68 \pm 2^{*,**}$	$56 \pm 5^*$	$22 \pm 2^{*,**}$

Values are mean $\pm$ SEM. Oscillating cells were defined as having Ca^{2+} transients with an amplitude >20 nM. Baseline Ca^{2+} was defined as the lowest Ca^{2+} level during the recording period. Maximum amplitude indicates the peak amplitude of the Ca^{2+} transients relative to baseline. Average Ca^{2+} refers to the mean Ca^{2+} level above baseline during the recording period. Data were based on measurement of Ca^{2+} levels during collections of 1–5 min at 3–5 sec intervals.

*Significant difference compared with the corresponding value at P4 ($p < 0.05$; ANOVA; *post hoc* analysis by Fisher's protected least significant difference test).

**Significant difference compared with the corresponding value at P5 ($p < 0.05$; ANOVA; *post hoc* analysis by Fisher's protected least significant difference test).

example, that measured for spontaneous Ca^{2+} spikes (2.4 ± 0.3 spikes/hr) and waves (2.1 ± 0.2 waves/hr) in embryonic *Xenopus* spinal neurons (Gu et al., 1994) or for spontaneous Ca^{2+} fluctuations in the leading process of migrating cerebellar granule cells (13 ± 3 fluctuations/hr; Komuro and Rakic, 1996).

Statistics on various measures of the Ca^{2+} oscillations at different ages were obtained from collections of 1–5 min at 3–5 sec intervals. Measurements were made of baseline Ca^{2+} levels, the maximum amplitude of the Ca^{2+} oscillations relative to baseline, and the average Ca^{2+} level above baseline (Fig. 2B). The minimum amplitude (relative to baseline) for defining an oscillation was 20 nM. Approximately two-thirds (219 of 351 cells) of the P4 to P7 Purkinje neurons studied displayed oscillations in Ca^{2+} levels. In oscillating cells, age-related differences were observed in all measurements with the most prominent changes occurring between P4 and P5, when a significant increase in baseline Ca^{2+} and decrease in maximum amplitude and average Ca^{2+} were observed (Table 1). In nonoscillating cells, measurements were made of baseline Ca^{2+} levels, which showed no significant change between P4 and P7 (Table 1).

Nuclear Ca^{2+} changes

In the above studies, the intracellular Ca^{2+} levels represent an average of levels measured over the entire somata of the neurons. However, in a subpopulation of the neurons studied ($n = 20$) the nuclear and cytoplasmic regions were clearly distinguishable, enabling measurements of these two subcellular regions. This analysis indicated that a pattern of Ca^{2+} oscillations similar to that observed in the cytoplasmic region occurred in the nuclear region (Fig. 3). Baseline Ca^{2+} levels were typically lower ($24 \pm 4\%$) in the nucleus compared with the cytoplasm. However, the maximum amplitude of the Ca^{2+} oscillations in the nucleus and cytoplasm were comparable. Mean values for the 20 neurons studied were 103 ± 20 nM and 119 ± 28 nM for the maximum amplitude of the nuclear and cytoplasmic Ca^{2+} oscillations, respectively.

Generation of Ca^{2+} oscillations requires spontaneous electrical activity

Mature Purkinje neurons *in vivo* exhibit spontaneous electrical activity generated both by synaptic input and endogenous voltage-sensitive ionic mechanisms. This activity is expressed early in the developmental program (Woodward et al., 1969a,b; Gruol and Franklin, 1987). The acutely isolated Purkinje neurons are dissociated from their synaptic connections, making it unlikely that synaptic components contribute to the generation of the Ca^{2+} oscillations observed in the current study. However, several studies have shown that the endogenously generated component of the electrical activity is retained in acutely isolated Purkinje neurons (Nam and Hockberger, 1997; Raman and Bean, 1997, 1999). To determine whether the endogenously generated electrical activity plays a role in the generation of the Ca^{2+} oscillations, we tested the effect of TTX, which blocks the endogenously generated electrical activity of Purkinje neurons (Gruol and Franklin, 1987; Nam and Hockberger, 1997), on the Ca^{2+} oscillations. TTX ($1 \mu\text{M}$) com-

pletely abolished the Ca^{2+} oscillations, indicating an underlying involvement of Na^+ -dependent electrical activity in the generation of the Ca^{2+} transients (Fig. 4). TTX also blocked all spike activity recorded under current clamp (data not shown).

To examine the relationship between the spontaneous electrical activity and the Ca^{2+} oscillations, whole-cell (perforated patch), current-clamp recordings combined with Ca^{2+} imaging were performed on acutely isolated Purkinje neurons. In most cases, Purkinje neurons showing spontaneous Ca^{2+} oscillations were selected for study. The majority of Purkinje neurons studied ($n = 11$ of 13) exhibited sustained spontaneous electrical activity composed of single and doublet spikes with intervening quiescent periods of varying length, patterns similar to that reported by others for acutely isolated Purkinje neurons of similar postnatal age (Nam and Hockberger, 1997) and immature Purkinje neurons *in vivo* (Woodward et al., 1969a,b) and in culture (Gruol and Franklin, 1987). Spontaneous Ca^{2+} oscillations correlated closely with the spontaneous electrical firing pattern in the combined recordings (Fig. 5A; see also Fig. 7). Moreover, manipulation of the endogenous electrical activity by applying hyperpolarizing or depolarizing current (Fig. 5B) produced a corresponding alteration in the Ca^{2+} oscillations, whereas in nonoscillating neurons depolarization of the membrane potential to elicit electrical activity produced Ca^{2+} signals similar to the spontaneous Ca^{2+} oscillations. Sustained electrical activity elevated resting Ca^{2+} levels because of summation of Ca^{2+} signals (Fig. 5B).

Ca^{2+} oscillations require Ca^{2+} entry mediated by L-type VGCCs

The correlation of electrical activity with the Ca^{2+} oscillations suggests that Ca^{2+} influx plays a primary role in the generation of the Ca^{2+} signals. To test for a dependence on Ca^{2+} influx, extracellular Ca^{2+} was reduced from 1.5 mM to 20 μM with EGTA (1 mM) in the bath saline. The Ca^{2+} oscillations were completely blocked by the reduction of extracellular Ca^{2+} ($n = 3$ from two preparations; Fig. 6A), indicating that influx of Ca^{2+} was required for the oscillations.

The two major avenues for Ca^{2+} entry into the neuron are through ligand-gated channels permeable to Ca^{2+} or through VGCCs. We first determined whether Ca^{2+} was entering through NMDA receptors because at this stage of development Purkinje neurons express NMDA receptors (Garthwaite et al., 1987; Krupa and Crepel, 1989; Rosenmund et al., 1992), which may have been activated by glutamate released from granule neurons that are present in the cerebellar cell preparation (Fig. 1). However, the oscillations were not affected by blocking NMDA receptors either with the competitive inhibitor APV (50 μM ; $n = 2$) or with the open channel blocker MK801 (10 μM ; $n = 3$; data not shown). AMPA, GABA_A , and GABA_B receptors were also not involved in Ca^{2+} entry because antagonists for these receptors were routinely included in the bath saline (see Materials and Methods).

We then tested inhibitors specific for the three principal classes of VGCCs (P/Q-, N-, and L-types) to determine whether the Ca^{2+}

Figure 3. Cytoplasmic Ca^{2+} oscillations are communicated to the nucleus. *A*, Ca^{2+} dynamics in the Purkinje neuron soma. The top row shows digital ratio (380/340 nm) images at selected time points during the Ca^{2+} recording shown in *B*; a paler blue indicates a higher ratio value. The bottom row shows the corresponding digital 380 nm wavelength; warmer colors correspond to lower Ca^{2+} levels, and cooler colors to higher Ca^{2+} levels. Numbers below the images in *A* correlate with numbers above collection time points in the Ca^{2+} trace in *B*. *B*, Ca^{2+} oscillations occurring simultaneously in the cytoplasm and nucleus of a P7 Purkinje neuron. Images were collected at 3 sec intervals for 90 sec. The imaging software was used to outline regions of similar size in the nucleus (red; see inset) and cytoplasm (blue) of the digital Ca^{2+} images.

Figure 4. Spontaneous Ca^{2+} oscillations require Na^{+} channel activity. Representative example of the effect of the Na^{+} channel blocker TTX ($1 \mu\text{M}$) on the spontaneous Ca^{2+} oscillations of an acutely isolated Purkinje neuron. As shown in this cell, TTX completely blocks the Ca^{2+} oscillations.

oscillations were dependent on Ca^{2+} influx through VGCCs. For these studies, measurements were made of the average Ca^{2+} above baseline and the maximum amplitude of the Ca^{2+} oscillations under control conditions and after addition of the antagonist to the bath saline. The baseline Ca^{2+} level for a cell was defined as the lowest Ca^{2+} level occurring during either the control or antagonist treatment period. Antagonists were considered to be effective if they reduced the average Ca^{2+} of a cell by $>10\%$.

Other laboratories have shown that P-type VGCCs are the primary Ca^{2+} channels mediating Ca^{2+} influx in 2- to 3-week-old

Purkinje neurons (Regan, 1991; Mintz et al., 1992). However, in our studies on the P4 to P7 Purkinje neurons, the P/Q type VGCC blocker ω -agatoxin IVA ($0.1 \mu\text{M}$) was relatively ineffective (Fig. 6*B*), decreasing Ca^{2+} levels in only 3 of 13 Purkinje neurons. Mean values ($\pm$ SEM) for average Ca^{2+} in the three neurons sensitive to ω -agatoxin IVA were $69 \pm 19 \text{ nM}$ under control conditions and $38 \pm 17 \text{ nM}$ after addition of ω -agatoxin IVA to the recording saline, an $\sim 45\%$ decrease. Mean values for the maximum amplitude of the Ca^{2+} oscillations were similar under the two conditions, $169 \pm 32 \text{ nM}$ under control conditions and $161 \pm 36 \text{ nM}$ in the presence of ω -agatoxin IVA ($n = 3$). Similarly, the N-type Ca^{2+} channel blocker ω -conotoxin GVIA ($1 \mu\text{M}$) decreased Ca^{2+} levels in only 3 of 8 Purkinje neurons (Fig. 6*C*). Mean values for average Ca^{2+} in the three neurons were $27 \pm 6 \text{ nM}$ under control conditions and $18 \pm 3 \text{ nM}$ in the presence of ω -conotoxin GVIA, an $\sim 33\%$ decrease. Mean values for the maximum amplitude of the Ca^{2+} oscillations in the three neurons were $102 \pm 16 \text{ nM}$ under control conditions and $62 \pm 17 \text{ nM}$ in the presence of ω -conotoxin GVIA ($n = 3$), a 39% decrease.

Surprisingly, the most consistent effects were observed with the L-type VGCC antagonist nimodipine, which was an effective antagonist of the Ca^{2+} oscillations in 27 of 29 neurons (Fig. 7*A,C*). Nimodipine ($1 \mu\text{M}$) reduced both the average Ca^{2+} and the maximum amplitude of the Ca^{2+} oscillations in all 27 neurons tested (from 17 preparations between P4 and P7). In those 27 neurons, mean values for average Ca^{2+} were $40 \pm 6 \text{ nM}$ under control conditions and $16 \pm 4 \text{ nM}$ in the presence of nimodipine, a 60% decrease. Mean values for the maximum amplitude of the Ca^{2+}

Figure 5. Ca^{2+} dynamics correlate closely with patterns of electrical activity in immature Purkinje neurons. *A*, Combined recording of the spontaneous Ca^{2+} oscillations (top trace) and the electrical activity (middle trace) in a single Purkinje neuron recorded at rest (i.e., without applied current; bottom trace). Note that the Ca^{2+} oscillations correlate with the appearance of spontaneous action potentials. The arrows indicate a burst event in the electrical activity and the corresponding increase of intracellular Ca^{2+} . *B*, Combined Ca^{2+} imaging/current-clamp recording in another Purkinje neuron. This cell exhibited little change in electrical activity or Ca^{2+} levels at rest. However, action potentials and corresponding Ca^{2+} signals could be induced by injecting depolarizing current (5 pA), first in two 2 sec pulses and then in a 50 sec pulse. The elevated resting Ca^{2+} level during the 50 msec current injection reflects the summation of high-frequency Ca^{2+} peaks. For both cells, the dashed lines indicate intracellular Ca^{2+} levels of 60 nM and a membrane potential of -60 mV.

oscillations were 101 ± 14 nM under control conditions and 28 ± 4 nM in the presence of nimodipine ($n = 27$), a 72% decrease.

When the above data were separated by age, the sensitivity of the Ca^{2+} oscillations to nimodipine (as measured by the effects of nimodipine on average Ca^{2+}) decreased between P4 and P6, suggesting a developmental regulation of either the activity or expression of the L-type Ca^{2+} channel. Mean values for average Ca^{2+} at P4 were 36 ± 7 nM ($n = 9$) under control conditions and 12 ± 3 nM in the presence of nimodipine, a 67% decrease, whereas at P6 mean values for average Ca^{2+} were 42 ± 13 nM ($n = 8$) under control conditions and 24 ± 12 nM in the presence of nimodipine, a 43% decrease. In combined electrophysiological and Ca^{2+} -imaging experiments, electrical spiking continued in the presence of nimodipine, although Ca^{2+} oscillations were abolished within seconds of introducing the drug (Fig. 7C), confirming that although the Ca^{2+} patterns were directly determined by the electrical activity, the specific influx of Ca^{2+} mediated by the L-type VGCCs was fundamental and essential to generating the Ca^{2+} oscillations. Thus, L-type VGCCs appear to be the primary mediators of Ca^{2+} influx generating the Ca^{2+} oscillations in the P4 to P7 Purkinje neurons.

Consistent with a dominant role for L-type VGCCs in the generation of the Ca^{2+} oscillations, the L-type channel-specific agonist BayK-8644 ($1 \mu\text{M}$) enhanced Ca^{2+} levels in six of eight cells tested (Fig. 7B,D). For the six cells, mean values for average Ca^{2+} were 46 ± 19 nM under control conditions and 135 ± 16 nM in the presence of BayK-8644, a 193% increase. Mean values for the maximum amplitude of the Ca^{2+} oscillations were 107 ± 32 nM under control conditions and 246 ± 86 nM in the presence of BayK-8644 ($n = 6$), a 130% increase. These results are consistent with the known action of BayK-8644 in prolonging the open time of L-type VGCCs (Hess et al., 1984). In combined electrophysiology/ Ca^{2+} imaging experiments, addition of BayK-8644 ($1 \mu\text{M}$) to the recording chamber changed the pattern of electrical activity from single-spike to burst events, which correlated with a large sustained increase in the Ca^{2+} resting level resulting from summation of the Ca^{2+} oscillations (Fig. 7D).

Expression of VGCCs in developing Purkinje neurons

Because our pharmacological results showing a prominent role for L-type VGCCs in the early developing Purkinje neurons were

somewhat unexpected, it was important to determine the expression of the L-, P/Q-, and N-type VGCCs at these early developmental stages. Immunostaining of acutely dissociated P5 cerebellar preparations with antibodies against specific subunits of the P-, N-, and L-type VGCCs showed that all three classes of Ca^{2+} channels were expressed by P5 Purkinje neurons (Fig. 8A). Somatic immunostaining for the P/Q-type channel α_{1A} subunit was strong and uniform for a given neuron, but showed variation from one Purkinje neuron to another. P/Q-type staining extended into the developing apical dendrite when dendritic structure was present (Fig. 8B) ($n = 10$). Immunostaining for L-type Ca^{2+} channels was uniform over the soma, almost as intense as for the P/Q-type, and heterogeneous in intensity within a given neuronal population. In contrast with the P/Q-type antibody, the L-type antibody did not consistently stain the developing apical dendrite (Fig. 8B; $n = 12$), suggesting a predominately somatic role for L-type VGCCs in developing Purkinje neurons. Somatic immunostaining for the N-type VGCCs was much fainter but also uniform for a given neuron. Staining with the N-type antibody was too faint to clearly resolve the presence of dendritic staining.

Amplification of the Ca^{2+} signal by release of Ca^{2+} from intracellular Ca^{2+} stores

Ca^{2+} influx through VGCCs is known to be amplified by release of Ca^{2+} from intracellular stores in Purkinje neurons (Llano et al., 1994; Kano et al., 1995a; Gruol et al., 1996). To test for an involvement of intracellular Ca^{2+} stores in the Ca^{2+} oscillations of the immature Purkinje neurons, the neurons were incubated for 20 min in $10 \mu\text{M}$ dantrolene, an agent known to block release of Ca^{2+} from RyR-gated intracellular Ca^{2+} stores. Dantrolene decreased the amplitude of the Ca^{2+} oscillations in 8 of 10 cells (from three preparations; Fig. 9). In those eight neurons, mean values for average Ca^{2+} were 52 ± 12 nM under control conditions and 26 ± 10 nM in the presence of dantrolene, a 50% decrease. Mean values for the maximum amplitude of the Ca^{2+} oscillations were 91 ± 18 nM under control conditions and 39 ± 13 nM in the presence of dantrolene ($n = 8$), a 57% decrease. These results suggested that a large component of the Ca^{2+} transients originated from intracellular Ca^{2+} stores. To verify that point, we tested ryanodine, which at relatively high concentrations blocks Ca^{2+} release from RyR-

Figure 6. Spontaneous Ca^{2+} oscillations in developing Purkinje neurons require Ca^{2+} influx through VGCCs. *A*, The Ca^{2+} traces show a typical example of the effect of EGTA (1 mM) to block the Ca^{2+} oscillations in an acutely isolated Purkinje neuron. For these studies, EGTA was applied by bath exchange in physiological saline containing low Ca^{2+} (20 μM). *B*, Representative example of the Ca^{2+} oscillations in a Purkinje neuron before and after bath application of the P/Q-type VGCC antagonist ω -agatoxin IVA (0.1 μM). ω -Agatoxin IVA had little effect on the amplitude or frequency of the Ca^{2+} oscillations. *C*, ω -Conotoxin GVIA (1 μM) was also ineffective at blocking the Ca^{2+} oscillations as shown in this cell.

gated intracellular Ca^{2+} stores (Ehrlich et al., 1994). Ryanodine (100 μM ; 20 min incubation) depressed the Ca^{2+} oscillations in 20 of 30 cells (from two preparations). In the 20 cells, mean values for average Ca^{2+} were 28 ± 4 nM under control conditions and 10 ± 2 nM in the presence of ryanodine, a 64% decrease. Mean values for the maximum amplitude of the Ca^{2+} oscillations were 68 ± 9 nM under control conditions and 30 ± 9 nM in the presence of ryanodine ($n = 20$), a 56% decrease.

DISCUSSION

Results from the current study show that spontaneous oscillations in intracellular Ca^{2+} are a prominent feature of early developing Purkinje neurons and that the Ca^{2+} oscillations are elicited by endogenously generated electrical activity that is expressed by this neuronal type (Woodward et al., 1969a,b; Crepel, 1972; Gruol and Franklin, 1987; Gruol et al., 1991; Nam and Hockberger, 1997; Raman and Bean, 1997, 1999). The Ca^{2+} oscillations are initiated by influx of Ca^{2+} through L-type VGCCs, which is then amplified by release of Ca^{2+} from RyR-gated intracellular Ca^{2+} stores and transmitted to the nucleus, a result that may have implications for gene regulation during neuronal development. These observations

made with early developing Purkinje neurons contrast with the known predominant role of P-type VGCCs in Ca^{2+} signaling in the dendritic region of mature Purkinje neurons (Hillman et al., 1991; Usowicz et al., 1992).

Ca^{2+} oscillations have been reported to occur spontaneously in several neuronal types (Sorimachi et al., 1990; Yuste et al., 1992; Gu et al., 1994; Gomez et al., 1995; Gu and Spitzer, 1995; Wong et al., 1995; Komuro and Rakic, 1996; Owens and Kriegstein, 1998; Gomez and Spitzer, 1999), and in some cases have been shown to play a role in the developmental process. For example, developmental signals for neurotransmitter expression and growth cone migration in embryonic *Xenopus* spinal neurons are encoded in patterns of spontaneous intracellular Ca^{2+} transients (Gu et al., 1994; Gu and Spitzer, 1995; Gomez and Spitzer, 1999). Spontaneous Ca^{2+} oscillations also play a critical role in regulating the advancement of the leading process of migrating cerebellar granule neurons (Komuro and Rakic, 1996), as well as, of chick retinal ganglion cell growth cones (Gomez et al., 1995). In these neuronal types, the trigger for spontaneous Ca^{2+} oscillations is still under investigation. However, our combined Ca^{2+} imaging and electrophysiological recordings show that the Ca^{2+} oscillations observed in the early developing Purkinje neurons are generated by spontaneous electrical activity that is endogenously generated.

The ability of immature Purkinje neurons to endogenously generate electrical activity both in the immature and mature states has been documented for Purkinje neurons in acutely isolated cerebellar cell preparations (Nam and Hockberger 1997; Raman and Bean, 1997, 1999), in cerebellar culture preparations (Gruol and Franklin, 1987; Gruol et al., 1991), and *in vivo* as early as 2 d postnatal (Woodward et al., 1969a,b; Crepel, 1972). Ionic models involving “resurgent” sodium currents (Raman and Bean, 1997) or sodium “window currents” (Nam and Hockberger, 1997) have been proposed to account for this component of the electrical activity of Purkinje neurons. Synaptic input also contributes to the spontaneous activity of mature and developing Purkinje neurons *in vivo* and in culture and may play a role in the Ca^{2+} dynamics of early developing Purkinje neurons. However, this component could not be examined in the current study because the synaptic connections were removed during the isolation procedure.

Our results show that the patterns of spontaneous electrical activity and Ca^{2+} oscillations are tightly linked in the early developing Purkinje neurons and that clusters of action potentials produce peaks of Ca^{2+} . Moreover, a higher frequency of action potentials corresponds to a Ca^{2+} response of greater amplitude. Thus, the level of electrical activity of early developing Purkinje plays a critical role in defining the level of intracellular Ca^{2+} . Different patterns of spontaneous electrical activity were present within a population of the acutely isolated Purkinje neurons at a single age, presumably resulting from ongoing developmental processes. The pattern of spontaneous electrical activity in Purkinje neurons is known to change with the developmental program (Woodward et al., 1969a,b; Gruol and Franklin, 1987). Therefore, it is likely that the patterns of Ca^{2+} oscillations produced by the spontaneous electrical activity will also change during development.

Pharmacological analysis showed that the Ca^{2+} oscillations in the developing Purkinje neurons are generated by the entry of extracellular Ca^{2+} through L-type VGCCs. This was unexpected because the P-type channel blockers ω -agatoxin IVA and funnel web spider toxin almost completely inhibit Ca^{2+} currents in acutely isolated Purkinje neurons from 2- to 3-week-old rats (Mintz et al., 1992) and in the dendrites of mature Purkinje neurons (Usowicz et al., 1992), respectively, indicating the functional importance of P/Q-type VGCCs at these stages. The dihydropyridine agonist BayK-8644 did not enhance Ca^{2+} influx in mature Purkinje neurons (Usowicz et al., 1992), implying that L-type Ca^{2+} channels do not play a prominent functional role in the mature dendritic arbor. However, the N-type Ca^{2+} channel blocker ω -conotoxin GVIA inhibited 6%, and the L-type Ca^{2+} channel blocker nitrendipine inhibited 8% of the high-threshold Ca^{2+} currents in acutely isolated Purkinje neurons from 1- to 3-week-old rats (Regan, 1991),

Figure 7. L-type VGCCs mediate Ca^{2+} influx that generates intracellular Ca^{2+} oscillations in immature Purkinje neurons. *A*, Representative example of the effect of nimodipine ($1 \mu\text{M}$; added to the bath saline) to nearly block Ca^{2+} oscillations in an acutely isolated Purkinje neuron. *B*, Incubation of the cells with BayK-8644 ($1 \mu\text{M}$) dramatically enhanced the Ca^{2+} oscillations of oscillating Purkinje neurons. *C*, Combined Ca^{2+} -imaging, current-clamp experiment showing simultaneous inhibition of spontaneous Ca^{2+} oscillations and spontaneous electrical activity by nimodipine ($1 \mu\text{M}$). Nimodipine was applied to the bath saline at the arrow and was present throughout the remainder of the recording period. *D*, Combined Ca^{2+} imaging, current-clamp experiment showing the simultaneous effect of BayK-8644 ($1 \mu\text{M}$) on spontaneous Ca^{2+} oscillations and spontaneous electrical activity. BayK-8644 was applied to the bath saline at the arrow and was present throughout the remainder of the recording period. Recordings in *C* and *D* are at the resting membrane potential of the cells; dashed lines denote Ca^{2+} levels and membrane potential as indicated to the left of the lines. Insets in *C* and *D* show records of the spike activity at an expanded time scale.

indicating that both of these channel types contributed to Ca^{2+} influx in developing Purkinje neurons. In our experiments, spontaneous Ca^{2+} oscillations in P4 to P7 Purkinje neurons were strongly inhibited by nimodipine as well as enhanced by the dihydropyridine agonist BayK-8644. Together, these results indicate that functional L-type VGCCs are present on Purkinje cells at an early age, and that these Ca^{2+} channels play an important role in Ca^{2+} signaling in early development.

Intracellular Ca^{2+} stores in the endoplasmic reticulum represent a critical intermediary step in many neuronal Ca^{2+} signaling pathways. Our results show that early in development, a somatic Ca^{2+} signaling pathway links Ca^{2+} entering through L-type VGCCs to Ca^{2+} -mediated release of Ca^{2+} from the RyR-gated intracellular Ca^{2+} stores. A similar Ca^{2+} signaling pathway has been shown to generate spontaneous Ca^{2+} oscillations in the rat frontal cortex (Hayashi et al., 1997) and agonist-evoked Ca^{2+} signals in other neuronal types (Chavis et al., 1996; Ronde and Nichols, 1997). IP3R-gated intracellular Ca^{2+} stores could also contribute to the spontaneous Ca^{2+} signals observed in the current study, if ambient IP3 and Ca^{2+} levels were sufficient to activate this pathway. IP3-mediated release of Ca^{2+} from the IP3R-gated intracellular Ca^{2+} stores has been shown to be important in dendritic Ca^{2+} signaling in Purkinje neurons (Gruol et al., 1996; Netzeband et al., 1997; Finch and Augustine, 1998) in addition to Ca^{2+} release linked to

RyRs (Llano et al., 1994; Gruol et al., 1996). Future studies will address this issue.

Immunostaining by other laboratories has shown that all three types of high-threshold VGCCs (P/Q-, L-, N-) are present on mature Purkinje neurons (Ahljanian et al., 1990; Hillman et al., 1991; Westenbroek et al., 1992). Our results show that all three VGCC types are already expressed in the soma of immature Purkinje neurons by the first postnatal week. However, pharmacological analysis showed that P- and N-type Ca^{2+} channels do not play a prominent role in generating the Ca^{2+} oscillations and that L-type VGCCs were the predominant Ca^{2+} channel involved. This selective involvement of L-type VGCCs in the Ca^{2+} oscillations could reflect functional coupling between L-type VGCCs and RyR-gated intracellular Ca^{2+} stores, as has been shown to exist in other neuronal systems (Chavis et al., 1996). Such an association produces selective amplification of Ca^{2+} signals through L-type VGCCs. Alternatively, other features of the Ca^{2+} signaling system such as the topographic distribution of VGCCs or intracellular Ca^{2+} stores may underlie the prominent role of L-type VGCCs. Further studies will be necessary to resolve this issue.

Activation of L-type Ca^{2+} channels has been directly linked to second messenger pathways that lead to increased expression of immediate early genes in several neuronal cell types (Murphy et al., 1991; Bading et al., 1993; Deisseroth et al., 1998; Rajadhyaksha et

Figure 8. P/Q-, N-, and L-type VGCCs are expressed by immature Purkinje neurons. *A*, Photomicrographs showing immunoreactivity of early developing Purkinje neurons (arrows) for P/Q- (top, left panel), L- (middle, left panel), and N-type (bottom, left panel) VGCCs. P/Q-, L-, and N-type VGCCs were immunostained with antibodies to the α_{1A} , α_{1C} , and α_{1B} subunits of Ca^{2+} channels, respectively. Immunoreactivity is indicated by the brown color and was blocked in the presence of the corresponding blocking peptide (1:1 ratio of the antibody and peptide by weight) as shown in the panels to the right. All panels show P5 neurons. *B*, Photomicrographs demonstrating the relative expression of P/Q-type (left panel) and L-type (right panel) VGCCs in the developing apical dendrites (arrows) compared with the somata of immature Purkinje neurons. The dendrites typically expressed P-type VGCCs with relatively low levels of immunoreactivity for L-type VGCCs. Both panels show P7 neurons. Scale bars, 15 μm .

al., 1999). Our studies showing that electrically induced Ca^{2+} oscillations are transmitted to the nucleus suggest that such a pathway may be important for signaling in developing Purkinje neurons. Studies in other cell types have shown that cytosolic Ca^{2+} signals can be translated into changes in nuclear Ca^{2+} and that nuclear Ca^{2+} signals can also be initiated in the nucleus (Kocsis et al., 1994; Gerasimenko et al., 1996). Both RyRs and IP3Rs are present on the nuclear membrane (Kocsis et al., 1994; Gerasimenko et al., 1996; Humbert et al., 1996), a membrane that is formed by an extension of the endoplasmic reticulum. Nuclear Ca^{2+} has been shown to be capable of modulating transcription (Hardingham et al., 1997) and dynamic changes in the nuclear Ca^{2+} pool, including Ca^{2+} oscillations and bursts of Ca^{2+} transients have been shown to act as specific switches for increasing gene expression (Gu and Spitzer, 1995; Dolmetsch et al., 1998; Li et al., 1998), potentially by activating Ca^{2+} -sensitive intermediary

Figure 9. Ca^{2+} signals of extracellular origin are amplified by release of Ca^{2+} from intracellular Ca^{2+} stores. *A*, Representative examples of the Ca^{2+} oscillations observed in an immature Purkinje neuron at time 0 (0') and 20 min (20') later. Under control conditions there was little change in the amplitude or frequency of the Ca^{2+} oscillations over time. *B*, Typical Ca^{2+} traces showing the effect of the RyR antagonist dantrolene (10 μM ; 20 min incubation) to depress Ca^{2+} oscillations in another Purkinje neuron. *C*, Current-clamp recording of the spontaneous electrical activity in another Purkinje neuron showing the persistence of electrical spiking after the 20 min incubation with dantrolene (10 μM). Resting membrane potential (-64 mV) is indicated by the dashed line. *V_m*, Membrane voltage.

components such as the enzyme CaM kinase II (DeKoninck and Schulman, 1998) or the transcription factor cAMP response element-binding protein (Hardingham et al., 1997). Thus, Ca^{2+} oscillations generated by the coordinated action of spontaneous electrical activity, L-type voltage-sensitive Ca^{2+} channels, and RyR-gated intracellular Ca^{2+} stores may represent a developmental signal linking electrical activity at the neuronal cell membrane with changes in gene expression in developing Purkinje neurons.

REFERENCES

- Ahlijanian MK, Westenbroek RE, Catterall WA (1990) Subunit structure and localization of dihydropyridine-sensitive Ca^{2+} channels in mammalian brain, spinal cord, and retina. *Neuron* 4:819–832.
- Altman J (1972) Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. *J Comp Neurol* 145:399–464.
- Altman J, Bayer SA (1997) Development of the cerebellar system in relation to its evolution, structure and functions. New York: CRC.
- Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* 260:181–186.
- Baptista CA, Hatten ME, Blazeski R, Mason CA (1994) Cell-cell interactions influence survival and differentiation of purified Purkinje cells *in vitro*. *Neuron* 12:243–260.
- Chavis P, Fagni L, Lansman JB, Bockaert J (1996) Functional coupling between ryanodine receptors and L-type calcium channels in neurons. *Nature* 382:719–722.
- Crepel F (1972) Maturation of the cerebellar Purkinje cells. I. Postnatal

- evolution of the Purkinje cell spontaneous firing in the rat. *Exp Brain Res* 14:463–472.
- Deisseroth K, Heist EK, Tsien RW (1998) Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature* 392:198–202.
- De Koninck P, Schulman H (1998) Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. *Science* 279:227–230.
- Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 392:933–936.
- Ehrlich BE, Kaftan E, Bezprozvannaya S, Bezprozvanny I (1994) The pharmacology of intracellular Ca^{2+} -release channels. *Trends Pharmacol Sci* 15:145–149.
- Eilers JA, Augustine GJ, Konnerth A (1995) Subthreshold synaptic Ca^{2+} signaling in fine dendrites and spines of cerebellar Purkinje neurons. *Nature* 373:155–158.
- Eilers J, Plant T, Konnerth A (1996) Localized calcium signaling and neuronal integration in cerebellar Purkinje neurons. *Cell Calcium* 20:215–226.
- Enderlin S, Norman AW, Celio MR (1987) Ontogeny of the calcium binding protein calbindinD-28k in the rat nervous system. *Anat Embryol* 177:15–28.
- Finch EA, Augustine GJ (1998) Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. *Nature* 396:753–760.
- Garthwaite G, Yamini Jr B, Garthwaite J (1987) Selective loss of Purkinje and granule cell responsiveness to *N*-methyl-D-aspartate in rat cerebellum during development. *Dev Brain Res* 36:288–292.
- Gerasimenko OV, Gerasimenko JV, Tepikin AV, Peterson OH (1996) Calcium transport pathways in the nucleus. *Pflügers Arch* 432:1–6.
- Gomez TM, Spitzer NC (1999) *In vivo* regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* 397:350–354.
- Gomez T, Snow DW, Letourneau PC (1995) Characterization of spontaneous calcium transients in nerve growth cones and their effect on growth cone migration. *Neuron* 14:1233–1246.
- Gruol DL, Franklin CL (1987) Morphological and physiological differentiation of Purkinje neurons in cultures of rat cerebellum. *J Neurosci* 7:1271–1293.
- Gruol DL, Jacquin T, Yool AJ (1991) Single-channel K^{+} currents recorded from the somatic and dendritic regions of cerebellar Purkinje neurons in culture. *J Neurosci* 11:1002–1015.
- Gruol DL, Deal CR, Yool AJ (1992) Developmental changes in calcium conductances contribute to the physiological maturation of cerebellar Purkinje neurons in culture. *J Neurosci* 12:2838–2848.
- Gruol DL, Netzeband JG, Parsons KL (1996) Ca^{2+} signaling pathways linked to glutamate receptor activation in the somatic and dendritic regions of cultured cerebellar Purkinje neurons. *J Neurophysiol* 76:3325–3340.
- Gu X, Spitzer NC (1995) Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature* 375:784–787.
- Gu X, Olson EC, Spitzer NC (1994) Spontaneous neuronal calcium spikes and waves during early differentiation. *J Neurosci* 14:6325–6335.
- Hardingham GE, Hawla S, Johnson CM, Bading H (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature* 260–265.
- Hayashi T, Kagaya A, Takebayashi M, Oyamada T, Inagaki M, Tawara Y, Yokota N, Horiguchi J, Su T-P, Yamawaki S (1997) Effect of dantrolene on KCl - or NMDA -induced intracellular Ca^{2+} changes and spontaneous Ca^{2+} oscillation in cultured rat frontal cortical neurons. *J Neural Transm* 104:811–824.
- Hess P, Lansman JB, Tsien RW (1984) Different modes of Ca^{2+} gating behavior favored by dihydropyridine Ca^{2+} agonists and antagonists. *Nature* 311:538–544.
- Hillman D, Chen S, Aung TT, Cherksey B, Sugimori M, Llinas R (1991) Localization of P-type calcium channels in the central nervous system. *Proc Natl Acad Sci USA* 88:7076–7080.
- Hockberger PE, Tseng H-Y, Connor JA (1989) Fura-2 measurements of cultured rat Purkinje neurons show dendritic localization of Ca^{2+} influx. *J Neurosci* 9:2272–2284.
- Hockberger PE, Yousif L, Nam JSC (1994) Identification of acutely isolated cells from developing rat cerebellum. *NeuroImage* 1:276–287.
- Humbert J-P, Matter N, Artault J-C, Koppler P, Malviya AN (1996) Inositol 1,4,5-trisphosphate receptor is located to the inner nuclear membrane vindicating regulation of nuclear calcium signaling by inositol 1,4,5-trisphosphate. Discrete distribution of inositol phosphate receptors to inner and outer nuclear membranes. *J Biol Chem* 271:478–485.
- Kano M, Garaschuk O, Verkhratsky A, Konnerth A (1995a) Ryanodine receptor-mediated intracellular calcium release in rat cerebellar Purkinje neurons. *J Physiol (Lond)* 487:1–16.
- Kano M, Schneggenburger R, Verkhratsky A, Konnerth A (1995b) Depolarization-induced calcium signals in the somata of cerebellar Purkinje neurons. *Neurosci Res* 24:87–95.
- Kocsis JD, Rand MN, Lankford KL, Waxman SG (1994) Intercellular calcium mobilization and neurite outgrowth in mammalian neurons. *J Neurobiol* 25:252–264.
- Komuro H, Rakic P (1996) Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. *Neuron* 17:275–285.
- Konnerth A, Dreessen J, Augustine GJ (1992) Brief dendritic calcium signals initiate long-lasting synaptic depression in cerebellar Purkinje cells. *Proc Natl Acad Sci USA* 89:7051–7055.
- Krupa M, Crepel F (1989) Transient sensitivity of rat cerebellar Purkinje cells to *N*-methyl-D-aspartate during development. A voltage clamp study in *in vitro* slices. *Eur J Neurosci* 2:312–316.
- Li W-H, Llopis J, Whitney M, Zlokarnik G, Tsien RY (1998) Cell-permeant caged InsP_3 ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature* 392:936–941.
- Llano I, DiPolo R, Marty A (1994) Calcium-induced calcium release in cerebellar Purkinje cells. *Neuron* 12:663–673.
- Mason CA, Christakos S, Catalano SM (1990) Early climbing fiber interactions with Purkinje cells in the postnatal mouse cerebellum. *J Comp Neurol* 297:77–90.
- Milosevic A, Zecevic N (1998) Developmental changes in human cerebellum: expression of intracellular calcium receptors, calcium-binding proteins, and phosphorylated and nonphosphorylated neurofilament protein. *J Comp Neurol* 396:442–460.
- Mintz IM, Venema VJ, Swiderek K, Lee T, Bean BP, Adams ME (1992) P-type calcium channels blocked by the spider toxin ω -Aga-IVA. *Nature* 355:827–829.
- Miyakawa H, Lev-Ram V, Lasser-Ross N, Ross WN (1992) Calcium transients evoked by climbing fiber and parallel fiber synaptic inputs in guinea pig cerebellar Purkinje neurons. *J Neurophysiol* 68:1178–1189.
- Murphy TH, Worley PF, Baraban JM (1991) L-type voltage-sensitive calcium channels mediate synaptic activation of immediate early genes. *Neuron* 7:625–635.
- Nam SC, Hockberger PE (1997) Analysis of spontaneous electrical activity in cerebellar Purkinje cells acutely isolated from postnatal rats. *J Neurobiol* 33:18–32.
- Narasimhan K, Pessah IN, Linden DJ (1998) Inositol-1,4,5-trisphosphate receptor-mediated Ca mobilization is not required for cerebellar long-term depression in reduced preparations. *J Neurophysiol* 80:2963–2974.
- Netzeband JG, Parsons KL, Sweeney DD, Gruol DL (1997) Metabotropic glutamate receptor agonists alter neuronal excitability and Ca^{2+} levels via the phospholipase C transduction pathway in cultured Purkinje neurons. *J Neurophysiol* 78:63–75.
- Owens DF, Kriegstein AR (1998) Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. *J Neurosci* 18:5374–5388.
- Rajadhyaksha A, Barczak A, Macias W, Leveque J-C, Lewis SE, Konradi C (1999) L-type Ca^{2+} channels are essential for glutamate-mediated CREB phosphorylation and c-fos gene expression in striatal neurons. *J Neurosci* 19:6348–6359.
- Raman IM, Bean BP (1997) Resurgent sodium current and action potential formation in dissociated cerebellar Purkinje neurons. *J Neurosci* 17:4517–4526.
- Raman IM, Bean BP (1999) Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. *J Neurosci* 19:1663–1674.
- Regan LJ (1991) Voltage-dependent calcium currents in Purkinje cells from rat cerebellar vermis. *J Neurosci* 11:2259–2269.
- Ronde P, Nichols RA (1997) 5-HT_3 receptors induce rises in cytosolic and nuclear calcium in NG108–15 cells via calcium-induced calcium release. *Cell Calcium* 22:357–365.
- Rosenmund C, Legendre P, Westbrook GL (1992) Expression of NMDA channels on cerebellar Purkinje cells acutely dissociated from newborn rats. *J Neurophysiol* 68:1901–1905.
- Ross WN, Werman R (1987) Mapping calcium transients in the dendrites of Purkinje cells from the guinea pig cerebellum *in vitro*. *J Physiol (Lond)* 389:319–336.
- Sorimachi M, Morita Y, Nakamura H (1990) Possible regulation of the cytosolic-free calcium concentration by Na^{+} spikes in immature cerebellar Purkinje cells. *Neurosci Lett* 111:333–338.
- Sugimori M, Llinas RR (1990) Real-time imaging of calcium influx in mammalian cerebellar Purkinje cells *in vitro*. *Proc Natl Acad Sci USA* 87:5084–5088.
- Surmeier DJ, Stefani A, Foehring R, Kitai ST (1991) Developmental expression of a slowly-inactivating voltage-dependent potassium current in rat neostriatal neurons. *Neurosci Lett* 122:41–46.
- Tank DW, Sugimori M, Connor JA, Llinas RR (1988) Spatially resolved calcium dynamics of mammalian Purkinje cells in cerebellar slice. *Science* 242:773–777.
- Usovich MM, Sugimori M, Cherksey B, Llinas R (1992) P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. *Neuron* 9:1185–1199.
- Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP, Catterall WA (1992) Biochemical properties and subcellular distribution of an N-type calcium channel $\alpha 1$ subunit. *Neuron* 9:1099–1115.
- Wong ROL, Chernjavsky A, Smith SJ, Shatz CJ (1995) Early functional neural networks in the developing retina. *Nature* 374:716–718.
- Woodward DJ, Hoffer BJ, Lapham LW (1969a) Correlative survey of electrophysiological, neuropharmacological, and histochemical aspects of cerebellar maturation in the rat. In: *Neurobiology of cerebellar evolution and development* (Llinas R, ed), pp 725–741. Chicago: AMP.
- Woodward DJ, Hoffer BJ, Lapham LW (1969b) Postnatal development of electrical and enzyme histochemical activity in Purkinje cells. *Exp Neurol* 23:120–139.
- Yuste R, Peinado A, Katz LC (1992) Neuronal domains in developing neocortex. *Science* 257:665–669.