Altered Calcium Homeostasis in Cerebellar Purkinje Cells of Leaner Mutant Mice

LEONARD S. DOVE,1 SANG-SOEP NAHM,2 DAVID MURCHISON,1 LOUISE C. ABBOTT,2 AND WILLIAM H. GRIFFITH1

1Department of Medical Pharmacology and Toxicology, College of Medicine, Texas A&M University System Health Science Center, College Station 77843-1114; and 2Department of Veterinary Anatomy and Public Health, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843-4458

Received 14 October 1999; accepted in final form 6 March 2000

Dove, Leonard S., Sang-soep Nahm, David Murchison, Louise C. Abbott, and William H. Griffith. Altered calcium homeostasis in cerebellar Purkinje cells of leaner mutant mice. J Neurophysiol 84: 513–524, 2000. The leaner (tg<sup>a</sup>) mouse mutation occurs in the gene encoding the voltage-activated Ca<sup>2+</sup> channel α<sub>1A</sub> subunit, the pore-forming subunit of P/Q-type Ca<sup>2+</sup> channels. This mutation results in dramatic reductions in P-type Ca<sup>2+</sup> channel function in cerebellar Purkinje neurons of tg<sup>a</sup>/tg<sup>a</sup> mice that could affect intracellular Ca<sup>2+</sup> signaling. We combined whole cell patch-clamp electrophysiology with fura-2 microfluorimetry to examine aspects of Ca<sup>2+</sup> homeostasis in acutely dissociated tg<sup>a</sup>/tg<sup>a</sup> Purkinje cells. There was no difference between resting somatic Ca<sup>2+</sup> concentrations in tg<sup>a</sup>/tg<sup>a</sup> and wild-type (+/+) cells. However, by quantifying the relationship between intracellular Ca<sup>2+</sup> elevations and depolarization-induced Ca<sup>2+</sup> influx, we detected marked alterations in rapid calcium buffering between the two genotypes. Calcium buffering values (ratio of bound/free ions) were significantly reduced in tg<sup>a</sup>/tg<sup>a</sup> cells (584 ± 52) Purkinje cells relative to +/+ (1.221 ± 80) cells. By blocking the endoplasmic reticulum (ER) Ca<sup>2+</sup>-ATPases with thapsigargin, we observed that the ER had a profound impact on rapid Ca<sup>2+</sup> buffering that was also differential between tg<sup>a</sup>/tg<sup>a</sup> and +/+ Purkinje cells. Diminished Ca<sup>2+</sup> uptake by the ER apparently contributes to the reduced buffering ability of mutant cells. This report constitutes one of the few instances in which the ER has been implicated in rapid Ca<sup>2+</sup> buffering. Concomitant with this reduced buffering, in situ hybridization with calbindin D28k and parvalbumin antisense oligonucleotides revealed significant reductions in mRNA levels for these Ca<sup>2+</sup>-binding proteins (CaBP)s in tg<sup>a</sup>/tg<sup>a</sup> Purkinje cells. All of these results suggest that alterations of Ca<sup>2+</sup> homeostasis in tg<sup>a</sup>/tg<sup>a</sup> Purkinje cells may serve as a mechanism whereby reduced P-type Ca<sup>2+</sup> channel function contributes to the mutant phenotype.

INTRODUCTION

The neurological mutant mouse leaner is a useful model of cerebellar dysfunction and pathogenesis. The leaner (tg<sup>a</sup>) mutation lies in a splice donor consensus sequence on the gene encoding the Ca<sup>2+</sup> channel α<sub>1A</sub> subunit (Fletcher et al. 1996), the pore-forming subunit of P- and Q-type voltage-activated Ca<sup>2+</sup> channels (Gillard et al. 1997; Sather et al. 1993; Stea et al. 1994). The tg<sup>a</sup> mutation results in a dramatic reduction in P-type Ca<sup>2+</sup> channel function in cerebellar Purkinje cells (Dove et al. 1998; Lorenzon et al. 1998), where P-type channels contribute approximately 90% to the whole cell Ca<sup>2+</sup> current (Dove et al. 1998; Mintz et al. 1992a,b). The greatly diminished whole cell Ca<sup>2+</sup> current density is apparently mediated by a reduction in the single-channel open probability of the mutant P-type channel (Dove et al. 1998), rather than by a reduction in the expression of the protein (Lau et al. 1998). This reduction in P-type channel function may have profound effects on intracellular Ca<sup>2+</sup> elevations and the mechanisms of Ca<sup>2+</sup> homeostasis in leaner Purkinje cells.

Prominent calcium signaling is a well-established aspect of cerebellar Purkinje cell physiology. The control of intracellular calcium concentrations ([Ca<sup>2+</sup>]) in Purkinje cells is a dynamic process involving influx through voltage-activated channels, buffering and sequestration by Ca<sup>2+</sup>-binding proteins (CaBP)s and intracellular organelles, and release from intracellular inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and ryanodine-sensitive stores (Eilers et al. 1996). The synchronous action of these mechanisms can be observed following excitatory transmission onto Purkinje cells, when transient increases in [Ca<sup>2+</sup>] occur at both the dendritic and somatic levels (Eilers et al. 1995a,b). Elevations in [Ca<sup>2+</sup>]direct many Purkinje cell functions including the induction of plasticity at both excitatory (Konnerth et al. 1992; Sakurai 1990) and inhibitory (Kano et al. 1992; Llano et al. 1991) synapses. Calcium-mediated long-term synaptic depression (LTD) is believed to be induced by the convergence of parallel and climbing fiber inputs to the Purkinje synapses that combine to activate both Ca<sup>2+</sup> influx through voltage-activated Ca<sup>2+</sup> channels and Ca<sup>2+</sup> release from IP<sub>3</sub>-sensitive stores (Svoboda and Mainen 1999). It is recognized that a modest, spatially restricted portion of the dendritic Ca<sup>2+</sup> signal arises from IP<sub>3</sub> induced Ca<sup>2+</sup> release (Finch and Augustine 1998; Takechi et al. 1998) following parallel fiber activation. The remainder of the postsynaptic Ca<sup>2+</sup> elevations are thought to be mediated by voltage-activated Ca<sup>2+</sup> channels (Eilers et al. 1996), which is consistent with observations that depolarization-induced Ca<sup>2+</sup> channel activation leads to robust increases in Purkinje cell [Ca<sup>2+</sup>], (Kano et al. 1995b; Lev-Ram et al. 1992; Tank et al. 1988).

The magnitude and duration of [Ca<sup>2+</sup>] elevations following Ca<sup>2+</sup> influx or release is tightly regulated by efficient Ca<sup>2+</sup> buffering mechanisms. These mechanisms can be functionally

Address reprint requests to W. H. Griffith.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
categorized into two types: rapid buffers that immediately reduce the free Ca\textsuperscript{2+} to a fraction of that which entered the cytoplasm, thus limiting the peak free [Ca\textsuperscript{2+}]\textsubscript{i}, and slow buffers that are responsible for the decay of the Ca\textsuperscript{2+} transient and the restoration of baseline [Ca\textsuperscript{2+}]\textsubscript{i}. The activities of the various Ca\textsuperscript{2+} homeostatic mechanisms overlap and interact to produce rapid and slow buffering. Purkinje cells are proposed to possess a high capacity to rapidly buffer Ca\textsuperscript{2+} (Fierro and Llano 1996). Much of this Ca\textsuperscript{2+} buffering may be attributable to the high levels of CaBPs, such as calbindin and parvalbumin (Iacopino et al. 1990; Kosaka et al. 1993; Winsky and Kuznicki 1995), present in Purkinje cells, but also may involve significant Ca\textsuperscript{2+} uptake into intracellular organelles, such as the endoplasmic reticulum (ER) and mitochondria (Bertridge 1998). In this report, we compare Ca\textsuperscript{2+} buffering in homoygous leaner (tg\textsuperscript{1288}) Purkinje cells with that in wild-type (+/+ ) cells. We show that leaner Purkinje cells have a diminished Ca\textsuperscript{2+} buffering ability, which we attribute to reduced uptake by the ER and reduced CaBPs. These findings illustrate the impact that a native mutation of a Ca\textsuperscript{2+} channel gene can have on Ca\textsuperscript{2+} homeostatic mechanisms.

METHODS

Animals

Male and female wild-type (+/+ ) and heterozygous leaner (tg\textsuperscript{1288}) mice on the C57BL/6J background were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred to obtain either wild-type (+/+ ) or homozygous leaner (tg\textsuperscript{1288}) mice. Mice were maintained on a 12-h light/dark cycle with constant temperature (23–24°C), constant humidity (45–50%), and free access to food (Wayne rodent chow) and water. As tg\textsuperscript{1288} mice become extremely ataxic, they were supplemented with hand feeding from postnatal day 15–16 through postnatal day 50. Handling and care of the animals was in accordance with policies of Texas A&M University and the National Institute of Health.

Isolated cells

Individual cerebellar Purkinje cells were obtained by 18- to 30-day-old mice using methods described previously (Dove et al. 1998). Briefly, mice were decapitated under isoflurane anesthesia and their cerebella removed. Parasagittal cerebellar slices (450 \mu m) were cut on a McIlwain tissue chatter and held in an oxygenated sucrose solution containing (in mM) 248 sucrose, 26 NaHCO\textsubscript{3}, 10 glucose, 5 KCl, 2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, and 1 Na-pyruvate (pH 7.4). Slices were enzymatically treated in sucrose solution containing 1.0 mg/ml Protease type XXIII (Sigma, St. Louis, MO) for 20 min at 35–36°C.

To isolate individual Purkinje cells, cerebellar slices were transferred to Dulbecco’s modified Eagles medium (GIBCO, Grand Island, NY) and mechanically triturated through a series of fire-polished Pasteur pipettes. Isolated cells were dispersed onto the glass floor of a recording chamber pretreated with 0.1% Alicant blue solution to facilitate cell adhesion. The recording chamber was placed on the headstage of an inverted microscope (Axiovert 100, Zeiss) and the cells continuously perfused. Purkinje cells were identified morphologically by their large somata and single stump of apical dendrite.

Electrical recording

Whole cell patch-clamp recordings were performed with an Axopatch 200A amplifier using pCLAMP software (Axon Instruments, Foster City, CA). Patch electrodes were pulled from borosilicate glass (No. 7052, Garner Glass, Claremont, CA) on a Flaming/Brown micro-electrode puller (Sutter Instruments, Novato, CA). Electrodes were coated with wax to reduce stray capacitance and fire-polished to final resistances of 4–5 MO\textsubscript{2}. Cell capacitance was read directly from the potentiometer after the capacitance transients were nullified. Series resistance was compensated >75% and was adjusted as necessary throughout the course of recordings. Cells were voltage clamped at holding potential of ~60 mV, and Ca\textsuperscript{2+} currents were elicited by depolarizing voltage steps to ~10 mV. Different levels of Ca\textsuperscript{2+} influx were generated by varying the duration of the voltage steps. Intervals of 1–2 min between voltage steps allowed the resulting Ca\textsuperscript{2+} transients to return to baseline. Data were low-pass filtered at 1 kHz and were acquired at a sampling rate of 0.2–4 kHz.

Intracellular [Ca\textsuperscript{2+}] measurements

A dual excitation wavelength ratiometric microfluorimetry system was used to determine the spatially averaged [Ca\textsuperscript{2+}]\textsubscript{i}, in the somata of selected Purkinje cells loaded with fura-2 K\textsubscript{5}. The excitation field (approximately 10 \mu m diam) was smaller than the soma of all Purkinje cells and was centered to maximally occupy cells. Illumination was provided by a xenon arc lamp (Zeiss), and the excitation wavelength was alternated between 340 and 380 nm by means of a rotating (40 Hz) filter-wheel. The fluorescence signal was collected by a photomultiplier tube (Hamamatsu) with a 510- to 560-nm band-pass filter. The output of the photomultiplier tube (340 and 380 nm wavelength samples) was directed to an analog divider circuit where the ratio of f340 to f380 signals was calculated following subtraction of background and cellular autofluorescence at each wavelength. Background fluorescence was canceled by zeroing the fluorescent signals from the 340- and 380-nm channels in a cell-free field, and autofluorescence was subtracted by reducing the f340 and f380 signals by the average amount of fluorescence recorded from cells not loaded with fura-2. Autofluorescence of patch-clamped cells was <1% of the average value of the f380 signal with the 6.0% filter at baseline [Ca\textsuperscript{2+}], and was not different between the two genotypes. A neutral density filter (1.0 or 6.0%) was placed in the excitation pathway to prevent dye bleaching and saturation of the photomultiplier.

Procedures for the estimation of [Ca\textsuperscript{2+}] and estimation of \Delta[Ca\textsuperscript{2+}] have been described in detail previously (Murchison and Griffith 1998). Briefly, experimental fluorescent ratios were converted to [Ca\textsuperscript{2+}] using the equation

\[
[Ca^{2+}] = K_d R = \frac{(R - R_{\text{min}})}{R_{\text{max}} - R}
\]

where \(K_d\) is the dissociation constant for fura-2, \(B\) equals f380\textsubscript{max}/f380\textsubscript{min}, \(R_{\text{min}}\) equals f340/f380 at zero Ca\textsuperscript{2+}, \(R_{\text{max}}\) equals f340/f380 at high Ca\textsuperscript{2+}, and \(R\) equals f340/f380 measured experimentally (Grynkiewicz et al. 1985). For this report, \([Ca^{2+}]\), was estimated using in vivo calibrations with \(K_d = 230 \text{mM}, R_{\text{min}} = 0.10, R_{\text{max}} = 6.24,\) and \(B = 11.5\).

Calculation of buffering capacity

Calcium buffering capacity was calculated by employing a modification of the method of Hille and colleagues (Tse et al. 1994). The buffering value \(\beta\) was determined as the ratio of buffer-bound to free ion using the equation

\[
\frac{Ca^{2+}}{\nu} = \Delta[Ca^{2+}] = \Delta[Ca^{2+}] + \Delta[\beta Ca^{2+}]
\]

By this model Ca\textsuperscript{2+} is the integral of Ca\textsuperscript{2+} influx (charge from measured \(I_{\text{Ca}}\), \(\nu\) is cell volume, \(\Delta[Ca^{2+}]\), is the measured change in intracellular free Ca\textsuperscript{2+}, \(\Delta[Ca^{2+}]\) is the change in concentration of Ca\textsuperscript{2+} bound to endogenous buffers, and \(\Delta[\beta Ca^{2+}]\) is the change of
Ca\(^{2+}\) bound to exogenous buffers (fura-2). Rearranging the above equation gives

\[
\frac{\text{Ca}_{\text{int}}^{2+}}{(\text{Ca}^{2+})_i} = 1 + \frac{\Delta [\text{Ca}^{2+}]_i}{\Delta [\text{Ca}^{2+}]_b} + \frac{\Delta [\text{BCa}^{2+}]_i}{\Delta [\text{Ca}^{2+}]_b} = 1 + \beta_i + \beta_b = 1 + \beta
\]

where \(\beta\) is the sum of the endogenous buffering strength (\(\beta_b\)) and exogenous buffering strength (\(\beta_i\)). The slope of the \(\Delta [\text{Ca}^{2+}]_i\) versus \(\text{Ca}^{2+}\) entry plot is therefore the quantity

\[
\frac{1}{1 + \beta}
\]

The cellular \(\text{Ca}^{2+}\) buffering value (\(\beta\)) is therefore the reciprocal of the slope minus one.

A plot of \(\Delta [\text{Ca}^{2+}]_i\) versus \(\text{Ca}^{2+}\) entry was constructed for each cell assayed by delivering depolarizing steps of varying duration to yield several levels of \(\text{Ca}^{2+}\) entry while measuring the resulting \(\Delta [\text{Ca}^{2+}]_i\). Calcium entry was determined by integrating the \(\text{Ca}^{2+}\) current over time and normalizing for cell volume (\(v\)). Cell volume was approximated from the cellular capacitance, assuming the capacitance of a biological membrane to be 1 \(\mu F/cm^2\), realizing this to be an overestimate of accessible cell volume (Neher 1995). There was no difference in the capacitance of Purkinje cells from mutant or wild-type mice (Dove et al. 1998). Cells were included in our analysis only if they contained at least three data points in the linear portion of the \(\Delta [\text{Ca}^{2+}]_i\) versus \(\text{Ca}^{2+}\) entry plot. The linear portions of the individual and composite buffering curves (the slopes of which are approximately the reciprocals of the buffering values) were fit by linear regressions, while the supralinear portions not used in any calculations were fit visually.

The rate of rise of the \(\text{Ca}^{2+}\) transients was determined for each cell by dividing the peak \(\Delta [\text{Ca}^{2+}]_i\) by the time-to-peak and taking the average of the rates for the 100- and 200-ms voltage steps with \(\Delta [\text{Ca}^{2+}]_i > 40 \text{ nM} \) (Fig. 3A). Slow buffering was assessed by calculating recovery values (Murchison and Griffith 1998) that are normalized for the amplitudes of the \(\text{Ca}^{2+}\) transients and that take into account all processes tending to remove free \(\text{Ca}^{2+}\) from the cytosol without implying a linear rate. The recovery values are determined by dividing the time to recover (measured from the peak of the \(\text{Ca}^{2+}\) transient to the point where the fluorescent ratio record first crosses the prestimulus baseline) by the peak \(\Delta [\text{Ca}^{2+}]_i\) (Fig. 3B).

**Solutions and drugs**

Cells in the recording chamber were continuously perfused with a solution containing (in mM) 140 NaCl, 3 KCl, 2 CaCl\(_2\), 1.2 MgCl\(_2\), 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulphonate (HEPES) and 33 d-glucose (pH 7.4 with NaOH, 310–330 mOsm). Prior to whole cell recordings, the external solution was exchanged for a modified recording solution containing (in mM) 132 NaCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 HEPES, 33 d-glucose, 10 tetraethylammonium chloride (TEA-Cl), and 0.0005 tetrodotoxin (Calbiochem, La Jolla, CA; pH 7.4 with NaOH, 310–330 mOsm). Thapsigargin (Alomone Labs, Jerusalem, Israel) was dissolved in ethanol (0.08% final concentration) and added directly to the bath. The internal pipette solution contained (in mM) 110 Cs-acetate, 15 CsCl, 10 TEA-Cl, 20 HEPES, 4 ATP, and 0.1 GTP (pH 7.2 with CsOH, 290–310 mOsm) with 50 \(\mu M\) fura-2 \(K^{+}\)-spheres of cerebellar folia IV and VIII 1 x 10\(^6\) CPM/section) and mouse parvalbumin mRNA bases 68–112 and mouse parvalbumin mRNA bases 68–112.

**In situ hybridization**

In situ hybridization was performed as previously described (Lau et al. 1998). Coronal sections (12 \(\mu m\) of cerebellum from eight +/- and eight \(tg^{lo}\) mice (postnatal day 30) were cut using a cryostat and thaw-mounted onto gelatin-coated slides. Calbindin D28k and parvalbumin single-stranded oligonucleotide probes were complementary to mouse cerebellum calbindin D28k mRNA bases 231–263 (Nordquist et al. 1988) and mouse parvalbumin mRNA bases 68–112 (Zuhlke et al. 1989). The oligonucleotide probes were radiolabeled with \(^{35}\)S-DATP (Dupont NEN, Boston, MA) using terminal deoxynucleotidyl transferase (GIBCO). Standard sense-strand controls confirmed the probe specificity.

The sections were fixed with 4% formaldehyde in phosphate-buffered saline, acetylated in saline (0.9% wt/vol NaCl) containing 0.25% acetic anhydride and 0.1 M triethanolamine, dehydrated in graded ethanol and delipidated using cholorform. The hybridization buffer contained 10 x 10\(^6\) counts per minute (CPM)/ml oligonucleotide, 50% formamide, 10% dextran sulfate, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl-sulfate, 0.6 M NaCl, 80 mM Tris-HCl (pH 7.4), 4 mM ethylenediamine tetraacetic acid (EDTA), 0.1 M dithiothreitol, and 0.2 mg/ml heparin sulfate. Sections were hybridized overnight with oligonucleotide probe (0.25 x 10\(^6\) CPM/section) in a 37°C humid chamber, then the sections were washed four times with 2 x sodium chloride plus sodium citrate (SSC) (1 x SSC; 150 mM sodium chloride, 15 mM sodium citrate) and 50% formamide and twice in 1 x SSC. The sections were rinsed first in distilled water, then 70% ethanol and air dried.

Sections were exposed for 5–7 days at room temperature to BioMax MR film (Kodak, Rochester, NY) to reveal the radiographic signal, then the sections were dipped in NTB-2 emulsion (Kodak), diluted 1:1 with deionized water. After 3–6 wk of exposure in the dark at room temperature, the sections were developed in Kodak D-19 developer, fixed in Kodak fixer, counterstained lightly with thionin, and then coverslipped.

A standardized area of Purkinje cells was determined from captured images of thionin stained anterior and posterior cerebellar sections from four animals of each genotype. Using the public domain NIH Image 1.61 program (available at http://rsb.info.nih.gov/nih-image/), Purkinje cell somata were manually outlined to determine soma size. For each animal used to determine the soma size, a total of 40 Purkinje cells were measured from the vermis and hemispheres. No significant difference in Purkinje cell soma size between +/- and \(tg^{lo}\) mice was observed, and the modal soma size was used as the standardized cell margin.

The numbers of silver grains located over Purkinje cells were counted as previously described (Nakagawa et al. 1996) with minor modifications. Bright-field color images were captured with uniform brightness through a Hamamatsu video camera attached to an upright microscope (Axioplan 2, Zeiss). The images were converted to grayscale and the brightness readjusted to achieve a consistent threshold level. The standardized perimeter was applied to captured images, and only Purkinje cells that fit this template were counted. A total of 80 cells per animal were measured equally from the vermis and hemispheres. No significant difference in Purkinje cell soma size between +/- and \(tg^{lo}\) mice was observed, and the modal soma size was used as the standardized cell margin.

The number of silver grains located over Purkinje cells was counted as previously described (Nakagawa et al. 1996) with minor modifications. Bright-field color images were captured with uniform brightness through a Hamamatsu video camera attached to an upright microscope (Axioplan 2, Zeiss). The images were converted to grayscale and the brightness readjusted to achieve a consistent threshold level. The standardized perimeter was applied to captured images, and only Purkinje cells that fit this template were counted. A total of 80 cells per animal were measured equally from the vermis and hemispheres. No significant difference in Purkinje cell soma size between +/- and \(tg^{lo}\) mice was observed, and the modal soma size was used as the standardized cell margin.

The number of silver grains located over Purkinje cells was counted as previously described (Nakagawa et al. 1996) with minor modifications. Bright-field color images were captured with uniform brightness through a Hamamatsu video camera attached to an upright microscope (Axioplan 2, Zeiss). The images were converted to grayscale and the brightness readjusted to achieve a consistent threshold level. The standardized perimeter was applied to captured images, and only Purkinje cells that fit this template were counted. A total of 80 cells per animal were measured equally from the vermis and hemispheres. No significant difference in Purkinje cell soma size between +/- and \(tg^{lo}\) mice was observed, and the modal soma size was used as the standardized cell margin.

The number of silver grains located over Purkinje cells was counted as previously described (Nakagawa et al. 1996) with minor modifications. Bright-field color images were captured with uniform brightness through a Hamamatsu video camera attached to an upright microscope (Axioplan 2, Zeiss). The images were converted to grayscale and the brightness readjusted to achieve a consistent threshold level. The standardized perimeter was applied to captured images, and only Purkinje cells that fit this template were counted. A total of 80 cells per animal were measured equally from the vermis and hemispheres. No significant difference in Purkinje cell soma size between +/- and \(tg^{lo}\) mice was observed, and the modal soma size was used as the standardized cell margin.

**Statistical analysis**

In situ hybridization data were analyzed using an ANOVA for a two-factor experiment (SAS, SAS Institute) followed by Scheffé’s F-test for post hoc analysis (\(\alpha = 0.05\)). All other data were analyzed...
using two-way ANOVA or t-tests where appropriate. Statistical significance was based on \( P < 0.05 \), with all values reported as means ± SE.

**RESULTS**

**Resting \([Ca^{2+}]_i\)**

Basal \([Ca^{2+}]_i\) was assessed in acutely dissociated \(+/+/\) and \(tg^{la}/tg^{la}\) cerebellar Purkinje cells loaded with 50 \( \mu M \) fura-2 \( K^+\) via the patch pipette and in cells loaded with sufficient fura-2 AM to provide approximately the same fluorescent intensity as in patched cells. This concentration of fura-2 does not contribute much to the endogenous buffering capacity of Purkinje neurons (Fierro et al. 1998) and therefore should operate effectively as a \([Ca^{2+}]_i\) indicator (Murchison and Griffith 1998). Data were collected 5–10 min after establishment of the whole cell patch-clamp configuration or 40–50 min after wash out of fura-2 AM from the bath (to allow for intracellular deesterification). Loading of the fluorescent indicator was assessed by monitoring the increase in 380 nm fluorescence. Despite ample evidence for reduced \(Ca^{2+}\) influx in \(tg^{la}/tg^{la}\) Purkinje cells due to reduced P-type \(Ca^{2+}\) current (Dove et al. 1998; Lorenzon et al. 1998), we found no differences in resting somatic \([Ca^{2+}]_i\) between \(+/+/\) and \(tg^{la}/tg^{la}\) Purkinje cells. In patched cells, the values were 70.3 ± 3.4 nM (mean ± SE; \( n = 19 \)) and 71.8 ± 5.1 nM (\( n = 13 \)); and in unpatched cells they were 119.5 ± 8.3 nM (\( n = 10 \)) and 113.9 ± 4.4 nM (\( n = 20 \)) for \(+/+/\) and \(tg^{la}/tg^{la}\) cells, respectively. These levels of resting \(Ca^{2+}\) are slightly higher than the 25–40 nM previously estimated for Purkinje cells in slice preparations (Fierro et al. 1998; Kano et al. 1995a; Llano et al. 1994), but are consistent with levels observed in other neurons (Miller 1991; Murchison and Griffith 1998). Thus \(tg^{la}/tg^{la}\) Purkinje cells are able to maintain a normal basal \([Ca^{2+}]_i\) despite reduced \(Ca^{2+}\) entry.

**Calcium buffering capacity**

Cerebellar Purkinje cells are thought to have a high endogenous \(Ca^{2+}\) binding ratio (Fierro and Llano 1996) which is a measure of the ability to buffer elevations in \([Ca^{2+}]_i\). We investigated whether the reduced function of P-type \(Ca^{2+}\) channels in \(tg^{la}/tg^{la}\) Purkinje cells could affect the changes in intracellular calcium concentration (\(\Delta[Ca^{2+}]_i\)) induced by the activation of these channels. We addressed this question with combined whole cell voltage-clamp and fura-2 microfluorimetric recordings, as previously described (Murchison and Griffith 1998). Five to 10 min after establishment of the whole cell recording configuration, voltage-gated \(Ca^{2+}\) channels were recorded in conventional whole cell voltage-clamp configuration. Dialysis for 5–10 min preceded data collection. Fluorescence ratios (\(f_{340}/f_{380}\)) were converted to calcium concentrations as described in METHODS. The linear portion of the buffering curves shown below are depicted. Dashed lines show the baseline level and the scale bar is relative.

**FIG. 1.** Calculation of rapid calcium buffering values for wild-type (\(+/+/\)) and leaner (\(tg^{la}/tg^{la}\)) Purkinje cells. Cells were loaded with 50 \( \mu M \) fura-2 \( K^+\) via patch pipette and recorded in conventional whole cell voltage-clamp configuration. Dialysis for 5–10 min preceded data collection. Fluorescence ratios (\(f_{340}/f_{380}\)) were converted to calcium concentrations as detailed in METHODS. A: superimposed calcium current records are shown for representative \(+/+/\) and \(tg^{la}/tg^{la}\) cells. Voltage-activated calcium currents were elicited by depolarizations to −10 mV of varied duration from a holding potential of −60 mV. Voltage protocol is shown above currents. B: superimposed changes in \(f_{340}/f_{380}\) representing the alterations in somatic calcium are shown, which correspond to the above calcium currents. The shortest duration current corresponds to the smallest change in \(f_{340}/f_{380}\), and the longest current to the largest change. C: the same fluorescent ratio records are shown individually and on an expanded scale. Only those records comprising the linear portion of the buffering curves shown below are depicted. Dashed lines show the baseline level and the scale bar is relative. C: data from the same \(+/+/\) and \(tg^{la}/tg^{la}\) cells were used to construct plots of the change in intracellular calcium concentration (\(\Delta[Ca^{2+}]_i\)) vs. calcium entry. Calcium entry was calculated by integrating the current as described in METHODS. The linear portions of the plots are extended as dashed lines.
activated by depolarizing pulses of varying duration, and the accompanying Ca\(^{2+}\) influx and \(\Delta[Ca^{2+}]_i\) were measured, as detailed in METHODS.

Figure 1A shows Ca\(^{2+}\) currents elicited by voltage steps to \(-10\) mV from a holding potential of \(-60\) mV in acutely dissociated +/+ and \(t^{b/a}g^{a}\) Purkinje cells. As previously observed, there was a marked reduction in peak current amplitude in the \(t^{b/a}g^{a}\) cells (note the calibration scales). Figure 1B shows the corresponding change in fluorescence ratio (f340/f380) associated with the Ca\(^{2+}\) currents in Fig. 1A. The smaller of these same fluorescence ratios are shown separated and expanded in Fig. 1C. These are the same traces used to measure the linear portion of the buffering curves below. In Fig. 1D, the calculated Ca\(^{2+}\) entry is plotted against the peak \(\Delta[Ca^{2+}]_i\). For both the +/+ and \(t^{b/a}g^{a}\) cell, the plot contains an initial linear portion, followed by a clear break from linearity at larger levels of Ca\(^{2+}\) influx. This supralinearity is interpreted as an indication of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), a process that amplifies Ca\(^{2+}\) signals by the release of Ca\(^{2+}\) sequestered in ryanodine-sensitive intracellular stores (Llano et al. 1994; Verkhratsky and Shmigol 1996). Supralinearity was observed in plots from all +/+ and \(t^{b/a}g^{a}\) cells where \(\Delta[Ca^{2+}]_i\) reached threshold levels. Also, caffeine application caused \(\Delta[Ca^{2+}]_i\) in all mutant cells examined (\(n = 6\), not shown), presumably by the activation of caffeine-sensitive ryanodine receptors, as described previously for these cells (Kano et al. 1995a). This suggests that ER signaling functions are maintained in \(t^{b/a}g^{a}\) cells.

The linear portion of the plot was used to calculate the rapid Ca\(^{2+}\) buffering capability, with the reciprocal of the slope yielding the Ca\(^{2+}\) buffering value of the cell. For the +/+ cell depicted in Fig. 1D, the plot yielded a buffering value of 1,227, a large value consistent with previous characterization of Purkinje cells (Fierro and Llano 1996). In contrast, the \(t^{b/a}g^{a}\) cell in Fig. 1D possessed a much lower buffering value of 697. These values are typical of others in our analysis.

There was a significant reduction (\(P < 0.001\)) in average Ca\(^{2+}\) buffering values for \(t^{b/a}g^{a}\) Purkinje cells (584 \pm 52, \(n = 10\)) relative to +/+ (1,221 \pm 80, \(n = 11\)) as shown in Fig. 2B. Figure 2A plots cumulative data for all +/+ and \(t^{b/a}g^{a}\) cells included in our analysis. Despite the reduction in rapid buffering detected in the mutant cells, the mean rate of rise of the \(t^{b/a}g^{a}\) transients was significantly (\(P < 0.001\)) slower (196 \pm 21 nM/s, \(n = 10\)) than that of the +/+ cells (543 \pm 124 nM/s, \(n = 11\)). An example of this difference is shown in Fig. 3A. In the face of constant Ca\(^{2+}\) influx, a reduction in rapid buffering would be expected to increase the rate of rise of the intracellular Ca\(^{2+}\) signal. However, the reduced function of the \(t^{b/a}g^{a}\) P-type Ca\(^{2+}\) channel not only limits the peak amplitude of the Ca\(^{2+}\) transient, but also suppresses the rate of Ca\(^{2+}\) influx. Thus it appears that the rate of rise of an intracellular Ca\(^{2+}\) transient is critically influenced by the rate of Ca\(^{2+}\) influx. It is of interest to note in this regard, that the maximum Ca\(^{2+}\) current density, the P-type channel open probability (Dove et al. 1998) and the rate of rise of the Ca\(^{2+}\) transient in the \(t^{b/a}g^{a}\) neurons are each about one-third that of the +/+ cells, while the buffering values are only reduced about 50%. That the diminished influx rate in mutant Purkinje neurons does not appear to functionally limit the peak \(\Delta[Ca^{2+}]_i\) emphasizes the decrement in the ability of the mutant cells to buffer Ca\(^{2+}\).

Although we were specifically interested in obtaining data regarding the peak \(\Delta[Ca^{2+}]_i\) to assess rapid buffering, we also acquired information on the slow buffering Ca\(^{2+}\) clearance (return of Ca\(^{2+}\) transient to baseline) from some cells. In agreement with Fierro et al. (1998), we observed both fast and slow components of Ca\(^{2+}\) clearance, with the fast component becoming more prominent with increasing Ca\(^{2+}\) load. Because of this, the net rate of recovery increases with increasing amplitude of the Ca\(^{2+}\) transient. We therefore calculated recovery values according to our procedures in Murchison and Griffith (1998) for transients of \(<350\) nM amplitude (small load) and for transients of \(>350\) nM amplitude (large load). Examples of recovery from a small load are presented in Fig. 3B. For each cell for which recovery time information was available, we averaged the recovery values of all transients under 350 nM to obtain a single value and likewise for transients over 350 nM. The values for +/+ neurons were as follows: small load, 0.47 \pm 0.04 s/nM (\(n = 9\)); large load, 0.22 \pm 0.04 s/nM (\(n = 7\)). The values for large and small loads were significantly different (\(P < 0.001\)). These +/+ values were not significantly different from those of the mutant neurons: small load, 0.40 \pm 0.06 s/nM (\(n = 4\)); large load, 0.20 \pm 0.06 s/nM (\(n = 5\)).
previously sequestered Ca\textsuperscript{2+} one to three depolarizing pulses to deplete from the ER any following application of thapsigargin, cells were stimulated with of 100 ms for the \(1\) using this organelle more prominently. A decreased contribution of the ER to rapid buffering may account for the reduced Ca\textsuperscript{2+} buffering capacity of \(tg^{la}/tg^{la}\) Purkinje cells.

In addition to reducing buffering values, thapsigargin pre-treatment linearized the plot of \(\Delta [Ca^{2+}]_i\) versus Ca\textsuperscript{2+} entry for both genotypes. This is consistent with depletion of ER Ca\textsuperscript{2+} stores normally available for CICR, although the supralinearity of the control plots also may be partially explained by saturation of the ER buffering ability (see DISCUSSION). An alternative explanation of the dual effects of thapsigargin might be that blocking of the ER calcium pump raises the [Ca\textsuperscript{2+}], such that the concentration threshold for CICR is reached at lower levels of Ca\textsuperscript{2+} entry. In this scenario, the buffering values obtained from the \(\Delta [Ca^{2+}]_i\), versus Ca\textsuperscript{2+} entry plots would be skewed by the appearance of CICR from residual Ca\textsuperscript{2+} remaining in the ER. However, this is unlikely because thapsigargin had no effect on resting [Ca\textsuperscript{2+}], in either \(+/+, \) (68.2 ± 4.5 nM, \(n = 12\)) cells or \(tg^{la}/tg^{la}\) (78.7 ± 6.6 nM, \(n = 10\)) cells, and previously sequestered Ca\textsuperscript{2+} was depleted prior to collecting data. Because thapsigargin treatment is well known to delay the restoration of basal [Ca\textsuperscript{2+}], (Fierro et al. 1998; Murchison and Griffith 1998) and there was no difference between the recovery values of the \(+/+, \) and \(tg^{la}/tg^{la}\) cells, recovery in thapsigargin was not assessed.

Removal of ER buffering by thapsigargin also significantly increased the rate of rise of the Ca\textsuperscript{2+} transients in \(tg^{la}/tg^{la}\) neurons from 196 ± 21 nM/s (\(n = 10\)) to 300 ± 41 nM/s (\(n = 9\), \(P = 0.03\)), but not in \(+/+\) neurons (control: 543 ± 124 nM/s; thapsigargin: 720 ± 74 nM/s, \(n = 11\) for each). These data suggest that, in addition to reduced participation of the ER, some further buffering decrement may exist in mutant neurons. Because CaBPs are often considered to be mediators of rapid Ca\textsuperscript{2+} buffering, and CaBPs have been shown to control the rate of rise of Ca\textsuperscript{2+} transients (Chard et al. 1993), we determined the different level of mRNA expression of CaBPs between the two genotypes.

**Endoplasmic reticulum in rapid calcium buffering**

To address the differences in endogenous buffering capacity between the two genotypes, we first examined the contribution of the ER using thapsigargin, an irreversible inhibitor of the ER Ca\textsuperscript{2+} pump (Thastrup et al. 1990). Thapsigargin (400 nM) was bath-applied for 5–7 min and subsequently washed out, a treatment previously shown to effectively block ER calcium uptake (Murchison and Griffith 1998) without affecting voltage-gated Ca\textsuperscript{2+} channel function (Shmigol et al. 1995). Following application of thapsigargin, cells were stimulated with one to three depolarizing pulses to deplete from the ER any previously sequestered Ca\textsuperscript{2+} that might remain available for CICR, although in the absence of reloading, the ER stores are thought to spontaneously deplete within a few minutes (Borson et al. 1991). Thereafter, several levels of Ca\textsuperscript{2+} entry were generated as described above, and the accompanying \(\Delta [Ca^{2+}]_i\) was measured. Figure 4A shows the plots of \(\Delta [Ca^{2+}]_i\) versus Ca\textsuperscript{2+} entry for a \(+/+,\) and a \(tg^{la}/tg^{la}\) Purkinje cell pretreated with thapsigargin. For the \(+/+,\) cell, a buffering value of 187 was determined, while the \(tg^{la}/tg^{la}\) exhibited a buffering value of 182. Interestingly, buffering values following thapsigargin pretreatment were not significantly different for \(tg^{la}/tg^{la}\) Purkinje cells (301 ± 66, \(n = 9\)) relative to \(+/+,\) cells (377 ± 60, \(n = 11\)). However, for both genotypes thapsigargin pretreatment significantly reduced \((P < 0.001)\) buffering values relative to untreated controls (Fig. 4B), suggesting a major role for the ER in rapid Ca\textsuperscript{2+} buffering in Purkinje neurons. These data also imply that the ER is differentially involved in rapid buffering between the two genotypes, with \(+/+,\) Purkinje cells using this organelle more prominently. A decreased contribuTION OF THE ER TO RAPID BUFFERING MAY ACCOUNT FOR THE REDUCED CA\textsuperscript{2+} BUFFERING CAPACITY OF TG\textsuperscript{LA}/TG\textsuperscript{LA} PURKINJE CELLS.

The data also imply that the ER is differentially involved in rapid buffering between the two genotypes, with \(+/+,\) Purkinje cells using this organelle more prominently. A decreased contribution of the ER to rapid buffering may account for the reduced Ca\textsuperscript{2+} buffering capacity of \(tg^{la}/tg^{la}\) Purkinje cells.

In addition to reducing buffering values, thapsigargin pretreatment linearized the plot of \(\Delta [Ca^{2+}]_i\), versus Ca\textsuperscript{2+} entry for both genotypes. This is consistent with depletion of ER Ca\textsuperscript{2+} stores normally available for CICR, although the supralinearity of the control plots also may be partially explained by saturation of the ER buffering ability (see DISCUSSION). An alternative explanation of the dual effects of thapsigargin might be that blocking of the ER calcium pump raises the [Ca\textsuperscript{2+}], such that the concentration threshold for CICR is reached at lower levels of Ca\textsuperscript{2+} entry. In this scenario, the buffering values obtained from the \(\Delta [Ca^{2+}]_i\), versus Ca\textsuperscript{2+} entry plots would be skewed by the appearance of CICR from residual Ca\textsuperscript{2+} remaining in the ER. However, this is unlikely because thapsigargin had no effect on resting [Ca\textsuperscript{2+}], in either \(+/+,\) (68.2 ± 4.5 nM, \(n = 12\)) cells or \(tg^{la}/tg^{la}\) (78.7 ± 6.6 nM, \(n = 10\)) cells, and previously sequestered Ca\textsuperscript{2+} was depleted prior to collecting data. Because thapsigargin treatment is well known to delay the restoration of basal [Ca\textsuperscript{2+}], (Fierro et al. 1998; Murchison and Griffith 1998) and there was no difference between the recovery values of the \(+/+,\) and \(tg^{la}/tg^{la}\) cells, recovery in thapsigargin was not assessed.

Removal of ER buffering by thapsigargin also significantly increased the rate of rise of the Ca\textsuperscript{2+} transients in \(tg^{la}/tg^{la}\) neurons from 196 ± 21 nM/s (\(n = 10\)) to 300 ± 41 nM/s (\(n = 9\), \(P = 0.03\)), but not in \(+/+\) neurons (control: 543 ± 124 nM/s; thapsigargin: 720 ± 74 nM/s, \(n = 11\) for each). These data suggest that, in addition to reduced participation of the ER, some further buffering decrement may exist in mutant neurons. Because CaBPs are often considered to be mediators of rapid Ca\textsuperscript{2+} buffering, and CaBPs have been shown to control the rate of rise of Ca\textsuperscript{2+} transients (Chard et al. 1993), we determined the different level of mRNA expression of CaBPs between the two genotypes.

**Calcium-binding proteins**

It has been suggested that CaBPs of the “EF-hand” family, such as calbindin D28k and parvalbumin, may act as endogenous Ca\textsuperscript{2+} buffers in neuronal cells (Chard et al. 1993; Fierro and Llano 1996). We utilized in situ hybridization histochemistry to assess the levels of mRNA for these two CaBPs in coronal cerebellar sections from \(+/+,\) and \(tg^{la}/tg^{la}\) mice. Figure 5A shows high-power bright-field images of calbindin D28k mRNA hybridization for representative sections of \(+/+,\) and \(tg^{la}/tg^{la}\) cerebellum. In both genotypes, silver grains representing positive hybridization for calbindin D28k mRNA are present at the level of Purkinje cell somata. Figure 5B displays silver grains representing positive hybridization for parvalbumin in \(+/+,\) and \(tg^{la}/tg^{la}\) sections. Silver grains showing strong hybridization for parvalbumin mRNA were principally observed over Purkinje cell somata and to a lesser degree over somata in the molecular layer. Levels of mRNA for the two CaBPs were compared for individual \(+/+,\) and \(tg^{la}/tg^{la}\) Purkinje cells by quantifying silver grain density within the area of Purkinje cell somata as described in METHODS. Quantitative analyses of grain density were performed on high magnification images of cerebellar sections from \(+/+,\) \((n = 8)\) and \(tg^{la}/tg^{la}\), \((n = 8)\) mice. For both parvalbumin and calbindin D28k, mRNA levels were significantly reduced in Purkinje cells.
cells of tg16/tg16 mice, as shown in Fig. 6. For calbindin D28k mRNA, average grains per cell equaled 120 ± 2 for +/- mice and 98 ± 2 for tg16/tg16 mice (P < 0.001). The reduction in parvalbumin mRNA level was even more pronounced, with average silver grains per cell 274 ± 7 for +/- and 150 ± 4 for tg16/tg16 (P < 0.001). While corresponding protein levels were not determined, decreased mRNA levels for calbindin D28k and parvalbumin are consistent with the altered rate of rise of Ca2+ transients and reductions in Ca2+ buffering detected in tg16/tg16 Purkinje cells.

**DISCUSSION**

The leaner (tg16) mouse mutation occurs in the gene encoding the voltage-activated Ca2+ channel a1A subunit (Fletcher et al. 1996). Recent work by this laboratory (Dove et al. 1998) and others (Lorenzon et al. 1998) has demonstrated that this mutation leads to dramatic reductions in the function of P-type voltage-activated Ca2+ channels in cerebellar Purkinje cells of homozygous leaner (tg16/tg16) mice. P-type channels mediate roughly 90% of all voltage-activated Ca2+ current in Purkinje cells (Dove et al. 1998; Mintz et al. 1992a,b). In this report, we have described the consequences of reduced P-type voltage-activated Ca2+ channel function on the regulation of [Ca2+]i in tg16/tg16 Purkinje cells.

There was no difference in resting [Ca2+]i between tg16/tg16 and +/- Purkinje cells. Apparently normal resting [Ca2+]i in tg16/tg16 cells, despite reduced influx through P-type channels suggested that Ca2+ signaling might be modified in these mutant cells. By quantifying the relationship between Δ[Ca2+]i and Ca2+ influx, we detected a marked reduction in rapid Ca2+ buffering in tg16/tg16 Purkinje cells relative to +/- cells. For any given level of Ca2+ influx through voltage-gated Ca2+
channels, \( \text{tg}^{\text{la}}/\text{tg}^{\text{la}} \) cells exhibited somatic \( \text{Ca}^{2+} \) elevations of greater magnitude than those displayed by \(+/+\) cells. Likewise, a \( \text{Ca}^{2+} \) transient of similar amplitude is attained for a given stimulation, despite the diminished \( \text{Ca}^{2+} \) entry in the mutant neurons. This implies that reduced P-type channel function in \( \text{tg}^{\text{la}}/\text{tg}^{\text{la}} \) cells does not result in reduced \( \Delta \text{[Ca}^{2+}]_{\text{i}} \) following membrane depolarization, and that \( \text{Ca}^{2+} \) signaling processes may not be profoundly disrupted.

**Basis of altered \( \text{Ca}^{2+} \) homeostasis**

The regulation of \( \text{[Ca}^{2+}]_{\text{i}} \) is crucial to cellular physiology. Calcium ions control a variety of neuronal processes including transmitter release, cell excitability, and gene expression (Berridge 1998; Clapham 1995; Volpe et al. 1993). Modifications in \( \text{Ca}^{2+} \) regulation may represent important compensatory mechanisms initiated to maintain signaling function in \( \text{tg}^{\text{la}}/\text{tg}^{\text{la}} \) Purkinje cells. We believe that the reductions in rapid \( \text{Ca}^{2+} \) buffering that we have observed in the mutant cells probably represent compensatory homeostatic efforts to maintain normal \( \text{Ca}^{2+} \) signaling functions, such as CICR, despite greatly reduced \( \text{Ca}^{2+} \) influx through voltage-activated \( \text{Ca}^{2+} \) channels. Compensatory changes in \( \text{Ca}^{2+} \) buffering mechanisms are believed to occur during aging in other neurons (Murchison and Griffith 1998; Tsai et al. 1998), and during chronic depolarization of cultured neurons (Fickbohm and Willard 1999).

There is an intriguing possibility that this cellular attempt to provide normal \( \text{Ca}^{2+} \) signaling ultimately results in the death of the mutant neurons. It is well known that excessive \( \text{Ca}^{2+} \), particularly in mitochondria, can act as the trigger for neuronal death (Budd and Nicholls 1996; Nicotera and Orrenius 1998; Stout et al. 1998). The \( \text{tg}^{\text{la}}/\text{tg}^{\text{la}} \) animals that we examined were younger than 30 days, and so would not yet be expected to have suffered extensive loss of Purkinje cells (Herrup and Wilczynski 1982). However, older mutant animals suffer a dramatic loss of Purkinje neurons by a currently unknown mechanism (Heckroth and Abbott 1994). Our results suggest that reductions in both ER buffering and CaBPs may place a greater burden on mitochondrial \( \text{Ca}^{2+} \) buffering, which could in turn result in the induction of cell death through mitochondrial \( \text{Ca}^{2+} \) overload. The possibility that compensatory changes in the nervous system might eventually prove deleterious has been suggested also as a mechanism of age-related neuronal dysfunction (Cotman et al. 1995).

There is also evidence supporting an alternative explanation in which delayed maturation of Purkinje neurons in mouse cerebellar mutants results in the phenotype (Sotello 1990). In this scenario, the reduced rapid buffering ability of \( \text{tg}^{\text{la}}/\text{tg}^{\text{la}} \) cells is simply part of the continuum of delayed physiological
maturation that is presumably mediated by reduced P-type Ca\textsuperscript{2+} channel function in early development. Several lines of evidence support this possibility. Reduced Ca\textsuperscript{2+} buffering in the mutant Purkinje cells would be consistent with the developmental increase in buffering reported by Fierro and Llano (1996). Also, persistent multiple synaptic contacts on dendritic spines of tg\textsuperscript{la}/tg\textsuperscript{la} Purkinje cells and altered spinogenesis are reminiscent of the situation in immature +/- Purkinje cells (Rhyu et al. 1999). Additionally, tyrosine hydroxylase, which is known to be transiently expressed in early development of normal Purkinje cells, is persistently expressed in the mutant cells (Austin et al. 1992; Hess and Wilson 1991).

**Rapid Ca\textsuperscript{2+} buffering**

In situ hybridization histochemistry analysis revealed significant reductions in the levels of mRNA for the Ca\textsuperscript{2+} pumps calbindin D28k and parvalbumin in tg\textsuperscript{la}/tg\textsuperscript{la} cells. While mRNA levels may not directly reflect the expression of functional protein, our previous investigation of α1A Ca\textsuperscript{2+} channel mRNA in these cells revealed a relative correlation between levels of message and protein expression (Lau et al. 1998). Based on their presumed role as endogenous Ca\textsuperscript{2+} buffers, reductions in Ca\textsuperscript{2+} pumps in tg\textsuperscript{la}/tg\textsuperscript{la} Purkinje cells are consistent with the increased rate of rise of Ca\textsuperscript{2+} transients in the presence of thapsigargin, and with a possible role in the reduced buffering capacity of these cells. Interestingly, a Ca\textsuperscript{2+}-responsive element appears to control expression of calbindin D28k in Purkinje cells at the transcriptional level (Arnold and Heintz 1997). This mechanism has been proposed to alter the Ca\textsuperscript{2+} buffering capacity of these cells depending on Ca\textsuperscript{2+} loads. Likewise, expression of parvalbumin is up-regulated in neurons of the deep cerebellar nuclei in response to loss of Purkinje cell input in several mouse mutants that suffer Purkinje cell degeneration, including leaner (Baurle et al. 1998). This increase in parvalbumin could be a compensatory mechanism to enhance Ca\textsuperscript{2+} buffering in response to increased Ca\textsuperscript{2+} influx accompanying the increased excitation in cells of the cerebellar nuclei that are deprived of the tonic inhibitory input of Purkinje cells. Similar feedback mechanisms could operate to produce the compensatory changes in Ca\textsuperscript{2+} pumps proposed here. While Ca\textsuperscript{2+} pumps can profoundly alter the shape and amplitude of [Ca\textsuperscript{2+}], transients when transfected into or exogenously applied to cells (Chard et al. 1993; Lledo et al. 1992), the role these proteins play in Ca\textsuperscript{2+} buffering under physiological conditions remains largely unknown. Some insight has come from studies of calbindin D28k null mice, where postsynaptic Ca\textsuperscript{2+} transients in Purkinje cells are greater in magnitude than those observed in wild-type mice and have a larger rapidly decaying component (Airaksinen et al. 1997). This is consistent with a role for Ca\textsuperscript{2+} binding proteins as rapid buffers.

We have also observed a prominent role for the ER in Purkinje cell rapid Ca\textsuperscript{2+} buffering. Cerebellar Purkinje cells are known to express high levels of ER Ca\textsuperscript{2+} ATPases (Baba-Aissa et al. 1996b), including one isoform not expressed elsewhere in the CNS (Baba-Aissa et al. 1996a; Wu et al. 1995). These pumps allow the sequestration of Ca\textsuperscript{2+} into the ER lumen. A significant contribution of the ER to rapid Ca\textsuperscript{2+} buffering in Purkinje cells contradicts the presumption that the high endogenous buffering capacity of these cells is attributable solely to calcium-binding proteins (Fierro and Llano 1996). Surprisingly, the ER appears differentially involved in Ca\textsuperscript{2+} buffering between tg\textsuperscript{la}/tg\textsuperscript{la} and +/- Purkinje cells, as exclusion of this organelle from rapid buffering by inhibition of the Ca\textsuperscript{2+} ATPases yielded similar buffering capacities for the two genotypes. These data suggest that a lessened contribution of the ER may be the primary basis for the reduced rapid Ca\textsuperscript{2+} buffering observed in tg\textsuperscript{la}/tg\textsuperscript{la} Purkinje cells, with possible reductions in Ca\textsuperscript{2+} pumps complementing this change.

It might be anticipated that Purkinje cells with reduced Ca\textsuperscript{2+} pumps, like the tg\textsuperscript{la}/tg\textsuperscript{la} cells, would continue to show a decrement in rapid Ca\textsuperscript{2+} buffering ability relative to +/- cells, even in the presence of thapsigargin. However, block of ER buffering appears to account for almost the entire difference in the rapid buffering values of the two genotypes. There are several possible explanations for this observation involving the presumed interactions between the Ca\textsuperscript{2+} pumps and the ER. It should be emphasized, however, that the interactions of Ca\textsuperscript{2+} buffering mechanisms are not well understood, particularly in the case of Ca\textsuperscript{2+} pumps. The thapsigargin data suggest that even the reduced amount of Ca\textsuperscript{2+} pumps apparently present in the tg\textsuperscript{la}/tg\textsuperscript{la} neurons is sufficient to buffer the Ca\textsuperscript{2+} loads imposed in these experiments to a similar extent as the Ca\textsuperscript{2+} pumps present in the +/- cells. Thus it may be that the +/- Ca\textsuperscript{2+} pumps are present in considerable excess. The linearity of the Ca\textsuperscript{2+} buffering curves in thapsigargin shows that the non-ER rapid buffering mechanisms remain unsaturated in the presence of substantial Ca\textsuperscript{2+} loads. Alternatively, the apparent lack of additional rapid buffering deficit in the tg\textsuperscript{la}/tg\textsuperscript{la} cells treated with thapsigargin could be explained by increased activity of another rapid buffer, such as mitochondria. The primary impact of the reduced Ca\textsuperscript{2+} pumps in the mutant cells appears to be on the rate of rise of the Ca\textsuperscript{2+} transients. This was significantly increased after thapsigargin treatment, but not changed in the wild-type cells. This is consistent with the findings of Chard et al. (1993), showing that exogenous Ca\textsuperscript{2+} pumps were able to decrease the rate of rise of Ca\textsuperscript{2+} transients.

The ER has not been shown to be involved in the rapid buffering of peak Δ[Ca\textsuperscript{2+}], in other cell types. Block of the ER Ca\textsuperscript{2+} pumps by thapsigargin has generally been associated with a reduction in the slow buffering restoration of basal [Ca\textsuperscript{2+}] in neurons (Markram et al. 1995; Miller 1991; Murchison and Griffith 1998; Shmigol et al. 1994b, 1995). In the present study, thapsigargin affected rapid Ca\textsuperscript{2+} buffering as evidenced by an increased peak Δ[Ca\textsuperscript{2+}], for any given level of Ca\textsuperscript{2+} entry. Fierro et al. (1998) accorded the ER a role in slow buffering in Purkinje neurons based on evidence of prolonged Ca\textsuperscript{2+} transients in the presence of pump blockers, thapsigargin, and cyclopiazonic acid. However, these investigators had to use shorter duration whole cell depolarizations in the presence of the blockers to attain the same Ca\textsuperscript{2+} transient amplitude as in the controls, implying that there was some effect on peak Δ[Ca\textsuperscript{2+}], and thus on rapid Ca\textsuperscript{2+} buffering. These results support the hypothesis that the ER buffers Ca\textsuperscript{2+} differently in Purkinje cells than in other neuronal types. For instance, an investigation of rat basal forebrain neurons by this laboratory concluded that those cells had rapid buffering values of 200–400 and that the ER was involved primarily in slow buffering without obviously contributing to CICR (Murchison and Griffith 1998). In contrast, we find the Purkinje neurons to have much greater buffering values and an ER that is involved in rapid buffering and in robust CICR. There is an additional
implication of the interpretation of the ER as a rapid buffer with respect to the supralinearity of buffering curves observed in Purkinje and other cell types. While this has previously been considered to be due to CICR, there is also a possibility that some of the supralinearity is actually due to “saturation” of the ER buffering ability as the store becomes maximally filled, the slope of the supralinear line reflecting a combination of the buffering ability of the non-ER rapid buffers and the contribution of CICR. A similar explanation involving saturation of rapid buffers has been proposed recently for supralinear [Ca\(^{2+}\)]\(_i\) responses in the dendrites of Purkinje neurons where Ca\(^{2+}\) influx in the restricted dendritic space produces \(\Delta[\text{Ca}^{2+}]_i\) of tens of micromolar (Maeda et al. 1999).

**Nature of altered ER Ca\(^{2+}\) regulation**

Alterations in Ca\(^{2+}\) buffering by the ER have been reported elsewhere. For example, in rat adrenergic neurons, reduced ER buffering is thought to account for an age-related increase in norepinephrine release (Tsai et al. 1998). The nature of reduced ER buffering remains to be determined. The ER is probably partially full at rest (Garaschuck et al. 1997; Murcison and Griffith 1999; Shimigol et al. 1994a). It is conceivable that in the tg\(^{-}\)/tg\(^{+}\) Purkinje cells may be more fully loaded under resting conditions, lessening its capacity to sequester Ca\(^{2+}\). This might be expected for cells with reduced CaBPs. Not only would this place a greater burden on other rapid buffering mechanisms, in this case ER uptake, but also Ca\(^{2+}\) might be expected to spread further from sites of influx, thus enhancing the opportunities to fill the ER stores. Increased rapid buffering ability has been associated with decreased loading of ER Ca\(^{2+}\) stores in aged rat basal forebrain neurons (Murcison and Griffith 1999), and the opposite situation may pertain to Purkinje neurons of the mutant mouse. From the perspective of the +/+ Purkinje neurons, the full compliment of CaBPs would be expected to reduce the loading of the ER relative to that of the tg\(^{-}\)/tg\(^{+}\) cells, giving the wild-type ER a greater capacity to buffer Ca\(^{2+}\). Alternatively, the ER may merely be repositioned (Subramanian and Meyer 1997) more distal to calcium entry sites in tgl\(^{-}\)/tgl\(^{+}\) Purkinje cells.

As mentioned above, the ER is known to contribute to slow buffering in Purkinje and other neurons. Although we did not directly assess the relative contributions of mutant and wild-type ER to slow buffering, there was no change in the sum process of Ca\(^{2+}\) clearance between the two genotypes. This suggests that either the participation of the ER in slow buffering in the mutant cells is not disrupted as the involvement in rapid buffering is, or that other slow buffering mechanisms compensate by increased activity. Another explanation could involve the apparent reduction in the rapid buffering abilities of CaBPs. Because increased buffering by rapid buffers is known to slow Ca\(^{2+}\) clearance (Chard et al. 1993), a decrease in this buffering might enhance clearance and offset the diminished ER uptake. When clearance time is normalized to the amplitude of the Ca\(^{2+}\) transient, as in our lab’s method of calculating recovery values (Murcison and Griffith 1998), mouse Purkinje neurons show a pattern of slow recovery from small amplitude transients and relatively more rapid recovery following larger transients. We found the relative recovery to be about twice as fast for transients >350 nM, as for those below that concentration. Fierro et al. (1998) attributed an increased rapid decay of large amplitude transients in Purkinje cells to ER uptake. An increased contribution of the ER to Ca\(^{2+}\) buffering of large Ca\(^{2+}\) loads was proposed also in basal forebrain neurons, but the relative recovery of Ca\(^{2+}\) transients is significantly slowed with increasing transient amplitude in those cells (Murcison and Griffith 1998). The basis of this difference in the physiology of the two cell types is unknown, but it further emphasizes the contrasts between their Ca\(^{2+}\) homeostatic mechanisms.

In addition to contributing to Ca\(^{2+}\) buffering, Purkinje cell ER serves as an important reservoir of releasable Ca\(^{2+}\). Cultured Purkinje neurons provided some of the early evidence that the ER can function as a Ca\(^{2+}\) source or sink (Brorson et al. 1991); an interpretation that is now widely accepted as a general principle in neurons (Berridge 1998). The ER appears to be a continuous network (Martone et al. 1993) containing IP\(_3\) (Furuichi et al. 1993) and ryanodine receptors (Kuwajima et al. 1992), which mediate Ca\(^{2+}\) release (Pozzan et al. 1994; Verkhratsky and Shimigol 1996). Calcium release attributable to activation of these receptors is an important aspect of cell signaling (Clapham 1995) and appears to contribute to the induction of synaptic plasticity (Inoue et al. 1998; Kohda et al. 1995; Khodakhah and Armstrong 1997). For both +/+ and tgl\(^{-}\)/tgl\(^{+}\) Purkinje cells, we observed supralinearity in plots of \(\Delta[\text{Ca}^{2+}]_i\) versus Ca\(^{2+}\) entry, which was prevented by thapsigargin. As this is generally considered evidence of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Llano et al. 1994; Verkhratsky and Shimigol 1996), it would appear that alterations in Ca\(^{2+}\) homeostasis in the mutants preserve Ca\(^{2+}\) signaling processes of the ER. These results suggest that the reduced contribution of the ER to Ca\(^{2+}\) buffering in tgl\(^{-}\)/tgl\(^{+}\) Purkinje cells does not result from generalized ER dysfunction, but is part of an adaptive process to conserve function in a cell where the natural influx of Ca\(^{2+}\) is greatly reduced.

This work was supported in part by National Institutes of Health Grants AG-07805 to W. H. Griffith and NS-01681 to L. C. Abbott and by Texas A&M University Interdisciplinary Research Initiatives to W. H. Griffith and L. C. Abbott.

REFERENCES


Reduced calcium buffering in leaner mutant mice


Sakurai M. Calcium is an intracellular mediator of the climbing fiber in induction of cerebellar long-term depression. *Proc Natl Acad Sci USA* 87: 3383–3385, 1990.


