Upregulation of Calcium Homeostatic Mechanisms in Chronically Depolarized Rat Myenteric Neurons

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Fickbohm, David J. and Alan L. Willard. Upregulation of calcium homeostatic mechanisms in chronically depolarized rat myenteric neurons. J. Neurophysiol. 81: 2683–2695, 1999. Perturbations of intracellular Ca2+ ion concentration ([Ca2+]i) have important effects on numerous neuronal processes and influence development and survival. Neuronal [Ca2+]i is, in large part, dependent on activity, and changes in activity levels can alter how neurons handle calcium (Ca). To investigate the ability of neuronal Ca homeostatic mechanisms to adapt to the persistent elevation of [Ca2+]i, we used optical and electrophysiological recording techniques to measure [Ca2+]i transients in neurons from the rat myenteric plexus that had been chronically depolarized by growth in culture medium containing elevated (25 mM) KCl. When studied in normal saline, neurons that had previously been chronically depolarized for 3–5 days had briefer action potentials than control neurons, their action potentials produced by intracellular stores, or buffering and efflux that occurs after Ca2+ entry (Carafoli 1987; Kostyuk and Verkhratsky 1994; Miller 1991). For the purposes of this paper, the Ca homeostasis components are divided into two groups based on their time scale and effect on activity-dependent [Ca2+]i transients: fast Ca handling mechanisms, including Ca influx through Ca channels, Ca buffering by Ca-binding proteins, and Ca release from intracellular stores, act on the millisecond to second time scale to determine the amplitude of [Ca2+]i transients, whereas slow Ca handling processes, such as Ca sequestration and efflux, act over several seconds to control the recovery of [Ca2+]i transients.

Understanding how neurons adapt their Ca homeostatic capabilities in response to perturbations of [Ca2+]i is important for gaining insight into the ability of neurons to withstand potentially toxic challenges that can occur as a result of trauma, disease, or oxygen deprivation. In addition, knowledge of the processes underlying the adaptive capabilities of Ca homeostatic mechanisms may help us understand the changes that may occur during the aging of the nervous system. However, at present, relatively little is known about how (or whether) the regulation of the diverse cellular Ca handling mechanisms is coordinated. For example, what determines whether a neuron’s response to a physiologically significant perturbation of [Ca2+]i is altered influx, altered buffering, altered efflux, or some combination of these processes? To begin to address such questions, we have examined the ways in which neurons adjust their Ca homeostatic capacities in response to a sustained elevation of [Ca2+]i evoked by growing them in depolarizing concentrations of KCl.

Chronically depolarizing neurons by increasing the concentration of KCl in their culture medium is a convenient means of experimentally achieving long-term perturbations of [Ca2+]i. This manipulation is used commonly to enhance survival of a variety of central and peripheral neurons (Collins et al. 1991; Franklin et al. 1995; Galli et al. 1995; Nishi and Berg 1981; Scott 1971). The mechanism(s) by which chronic depolarization promotes neuronal survival is unknown. It may activate intracellular pathways that normally are activated by synaptic activity and/or by neurotrophic factors, both of which are eliminated or reduced when neurons are placed in cell cultures. In many cell types, chronic depolarization causes a long-term decrease in depolarization-evoked Ca influx through voltage-dependent Ca channels (Delorme and McGee 1986; Feron and Godfraind 1995; Ferrante et al. 1991; Franklin et al. 1992; Liu et al. 1994). Chronic depolarization likely mimics the effects of electrical activity, which also causes a long-lasting reduction in Ca current in vivo and in vitro (Hong and
with a CCD video camera (CCD 72, Dage-MTI). Because the purpose of our experiments was to accurately monitor changes in \([\text{Ca}^{2+}]_i\), the dynamic range of the imaging setup was adjusted so that cytoplasmic \([\text{Ca}^{2+}]_i\) signals occupied the maximum range possible. This procedure had implications for \([\text{Ca}^{2+}]_i\), calibration as discussed in the following text. Images (4- or 8-frame averages/wavelength) were digitized by Image-1/FL hardware and software (Universal Imaging, West Chester, PA). Video images usually were captured every 3.5 s. For a temporal resolution of 1.5–1.6 s/ratio, individual wavelength data (4- or 8-frame averages) were written to files without capturing images. Ratio images were formed in Image-1/AT (Universal Imaging) and converted to \([\text{Ca}^{2+}]_i\) images using calibration values determined as described in a later section. The \([\text{Ca}^{2+}]_i\), for each cell in an image was averaged for a region of its soma.

**Dye loading**

Cells were incubated for 30 min at 37°C in a 5% CO₂ atmosphere in 1 ml Eagle’s minimum essential medium (supplemented with 20 mM KCl for 25K cells) containing 3 μM fura-2 AM, 0.3% dimethyl sulfoxide (DMSO), and 200 μg/ml Pluronic. After incubation with fura-2 AM, the 5K or 25K cells were rinsed with 5K or 25K standard external solution, respectively, and incubated at room temperature, in darkness, for ≥15 min. 5K and 25K neurons achieved similar levels of fluorescence, indicating that overall dye loading was similar in the two groups. There was no obviously discernible difference in fura-2 localization in the two groups.

\[ [\text{Ca}^{2+}]_i \text{ calibration } \]

Conversion of video images to images of estimated \([\text{Ca}^{2+}]_i\), was done in Image-1/FL. For measurements of electrically stimulated \([\text{Ca}^{2+}]_i\), transients, we adjusted the camera, intensifier, and gain settings on a cell-by-cell basis to optimally use the linear range of the camera; signals from electrically evoked \([\text{Ca}^{2+}]_i\), transients typically occupied >80% of the system’s dynamic range. Therefore measurement of \(R_{max} (F_{340}/F_{380} \text{ at saturating Ca})\) (Grynkiewicz et al. 1985) was not possible because \(F_{340}\) exceeded the fluorescence intensity limit of the system. We used a standard curve generated with commercially obtained stock solutions (Calcium Calibration Kit II, Molecular Probes, Eugene, OR) to calibrate our system. The calibration solutions consisted of mixtures of \(K\_EGTA\) and \(Ca\_EGTA\), yielding 0, 17, 38, 65, 100, 150, 225, and 351 mM free \(\text{Ca}^{2+}\) in 100 mM KCl and 10 mM MOPS, pH 7.2 (10 mM EGTA final concentration). The ionic concentrations of the calibration solutions were not matched to concentrations in situ (see following text). Droplets (0.5 μl) of the calibration solutions and fura-2 pentapotassium salt were placed beneath H₂O-saturated mineral oil on glass or ACLAR coverslips as appropriate. The fura-2 pentapotassium salt concentrations ranged from 5 to 25 μM, depending on the apparent amount of dye loading in the cells and the camera and intensifier shutter settings and the gain settings used to image the cells. The ratio of the emitted intensities for 340 nm (360 nm for experiments involving estimation of Ca binding capacity, see following text) and 380 nm excitation (\(F_{340}/F_{380}\)) was calculated for each \(\text{Ca}^{2+}\) concentration. The ratios for the calibration standards were fitted to curves by linear interpolation and the estimated \([\text{Ca}^{2+}]_i\), values in cells were extrapolated from the curves. Because the purpose of these experiments was to compare Ca homeostasis of control and chronically depolarized cells rather than to determine the “true” absolute value of \([\text{Ca}^{2+}]_i\), we did not correct for differences in the behavior of fura-2 in situ and in buffered in vitro solutions. As discussed in Franklin et al. (1992), we have made extensive efforts to use in situ calibration protocols, but we find such methods to be very unreliable for cultured myenteric neurons. Nevertheless the in vitro calibration method provided a reliable standard and yielded consistent estimates of \([\text{Ca}^{2+}]_i\), changes in similarly treated cells. Accordingly, although we do not wish to imply that we

**Methods**

**Cell culture**

Myenteric neurons from the small intestines of Sprague-Dawley rat pups were grown in cell cultures as described previously (Franklin and Willard 1993; Nishi and Willard 1985) except that the cells were grown on glass or ACLAR (Allied Signal Plastics, Pottsville, PA) coverslips coated with poly-lysine and laminin instead of collagen-coated plastic. When chronically depolarizing neurons, KCl was added to raise the final concentration to 25 mM; these are called “25K cells.” The concentrations of other ions were not reduced to compensate for osmolarity changes caused by raising the KCl concentration. The effects of elevating the concentration of KCl do not appear to be due to changes in osmolality because elevating the NaCl concentration by an additional 20 mM has no effect on neuronal survival, expression of voltage-dependent Ca currents, or voltage-dependent K currents (Willard, unpublished data). 25K cells remain depolarized for years and have modulation contrast optics (>40 objective).

\[ [\text{Ca}^{2+}]_i \text{ measurement instrumentation } \]

\([\text{Ca}^{2+}]_i\), was estimated using fura-2 (Grynkiewicz et al. 1985). Cells were illuminated with light from a 75-W xenon lamp that passed through 340- and 380-nm band-pass excitation filters in a computer-controlled filterwheel (Metaltek Instruments, Research Triangle Park, NC) and neutral density filters in a second filterwheel (to minimize photobleaching). The light was transmitted by a quartz fiber optic light scrambler (G. W. Ellis, Woods Hole, MA) to an inverted microscope, reflected by a dichroic mirror and focused onto cells with a ×100 objective (Nikon Fluor, NA 1.3). Emitted light was filtered (510 nm), intensified (GenIIsys, Dage-MTI, Michigan City, IN), and imaged
have determined the absolute values of [Ca\(^{2+}\)], values for changes in [Ca\(^{2+}\)], are reported in units of nanomolar.

**Evoking [Ca\(^{2+}\)] transients with field electrodes**

Neuronal [Ca\(^{2+}\)], transients were elicited by field stimulation with a pair of platinum wire electrodes. The wires were 10 mm long and were separated by 6 mm. The wires were positioned just above the coverslip, and the stimulated cells were centered in the field. A Grass SIU5 stimulus isolation unit and a Grass S88 stimulator were used to deliver either single 150-V, 2.5-ms pulses or 25-Hz trains of 2–16 pulses. A similar system for stimulation has been reported to effectively evoke action-potential (AP)-induced [Ca\(^{2+}\)], transients in cultured rat hippocampal neurons (Jacobs and Meyer 1997).

**Electrophysiology**

Tight-seal whole cell recording techniques and tight-seal perforated-patch recording techniques were used. There were no significant differences between values obtained with the two recording methods, and the results generally are reported without reference to the exact method used. Patch pipettes were made from 1.5-mm Kimax-51 glass capillary tubing (Fisher) with tips and shanks coated with dental wax (Kerr Sticky Wax, Emeryville, CA). Tips were heat-polished and had resistances of 2–6 MΩ when filled with recording solutions. Such electrodes typically formed 2–5 GΩ seals with myenteric neuronal membranes. The higher resistance electrodes were used for experiments in which fura-2 was introduced to slow the rate of diffusion into the cells. The bathing solution was grounded via a 0.9% NaCl agar bridge. Experiments were performed at room temperature (20–24°C). An Axopatch-1D amplifier with a unity gain CV-4 headstage was used for both current- and voltage-clamp experiments. Current and voltage protocols were controlled by a microcomputer connected to the amplifier via a LabMaster TL-1-125 DMA interface. pClamp (versions 5.5 and 6.0, Axon Instruments, Foster City, CA) was used for data acquisition and analysis. APs evoked by injecting rectangular depolarizing current pulses were low-pass filtered at 2 kHz and sampled at 4 or 20 kHz. When necessary, current was injected to keep the membrane potential at −60 mV. Voltage commands modeled on APWs (‘action potential waveforms’; APWs) were used to elicit calcium currents (I\(_{\text{Ca}}\)). An APW consisted of a depolarizing ramp from −60 to +35 mV, followed by a biphasic repolarizing ramp to −60. Two APWs were used, with the following parameters (times in ms): 10.5 total duration, 1.5 rise time, 6.0 80% fall time, and 3.0 remaining fall time and 11.7 total duration, 1.5 rise time, 6.8 80% fall time, 3.4 remaining fall time. I\(_{\text{Ca}}\) elicited by APWs were filtered at 1 kHz and sampled at 38 kHz. In combined electrophysiological and [Ca\(^{2+}\)] measurements, I\(_{\text{Ca}}\) was evoked by depolarizing voltage-clamp steps. 5K cells were held at −60 mV and stepped to 0 mV for 5–200 ms. Because of their lower Ca currents densities, it was usually necessary to hold 25K cells at −90 mV to evoke Ca currents with charge densities similar to those of 5K neurons. However, holding potentials of −60 mV were used for 25K cells with large I\(_{\text{Ca}}\) and no differences in Ca handling capacities were noted. A P4 subtraction procedure (using quarter scale hyperpolarizing voltage commands) was used to correct for linear leakage currents for step depolarizations and APW commands. Ca\(^{2+}\) influx charge density was calculated by dividing the time integral of I\(_{\text{Ca}}\) by the membrane capacitance, an estimate of membrane area. Membrane capacitance was estimated by integrating the first 1.75 ms of capacitative transients (filtered at 10 kHz and sampled at 20 kHz) elicited by 20-mV hyperpolarizing steps from a holding potential of −60 mV. The membrane capacitance of the cells used for these experiments was not significantly altered by chronic depolarization (data not shown). This method of controlling for cell size was used instead of cell volume measurements due to the difficulty of measuring volume in myenteric neurons, which have irregular shapes.

**Estimation of endogenous Ca\(^{2+}\) buffer concentration**

Two methods, both of which use the properties of an exogenously introduced Ca binding agent, fura-2, to estimate the endogenous Ca buffer and its properties, were used to compare the endogenous Ca binding capacity of 5K and 25K neurons (Neher and Augustine 1992). Fura-2 was introduced into cells via tight-seal whole cell recording pipettes filled with cesium-based internal solution plus 500 µM fura-2 pentapotassium salt without any additional Ca\(^{2+}\) buffering agent. Cell fluorescence was imaged and measured as described earlier except 360 nm excitation was substituted for 430 nm and the dynamic range of the imaging setup was adjusted to allow measurement of R\(_{\text{max}}\) at saturating Ca levels. Small pipette tips (6 µM) were used to slow fura-2 entry into cells. The cytoplasmic concentration of fura-2 (B\(_{1}\)) and the loading time course were estimated from the loading curve of background-corrected, emitted light intensity at the isobestic, 360-nm excitation, wavelength, F\(_{360}\) (directly proportional to B\(_{1}\)). [Ca\(^{2+}\)] was estimated from F\(_{360}\)/F\(_{380}\), after subtraction of the background fluorescence from the fura-2-filled pipette, measured before going whole cell. F\(_{360}\) and F\(_{380}\) were recorded every 1.5–1.6 s per wavelength pair (4 frame averages). The Ca\(^{2+}\) buffer capacity of the fura-2 that had entered a cell, K\(_{\text{B}}\), was calculated from Eq. 31 of Neher and Augustine (1992). K\(_{\text{B}}\), the Ca\(^{2+}\)-binding constant of fura-2 (the inverse of K\(_{\text{B}}\)), was calculated using Eq. 5 of Gryniewicz et al. (1985). Values of K\(_{\text{B}}\) determined from 16.7, 37.6, 64.5, 100, 150, and 225 nM and 40 µM Ca\(^{2+}\) were averaged to yield an estimated K\(_{\text{B}}\) of 0.005 nM\(^{-1}\).

The ‘kinetic’ method (method 1 of Neher and Augustine 1992) analyzes changes that occur in the decay time constants (τ) of [Ca\(^{2+}\)], transients in a population of cells as the concentration of fura-2 (B\(_{1}\)) increases. Extrapolation back to the decay time constant in the absence of fura-2 yields an estimate for the endogenous Ca binding capacity of that population of cells. This method of analysis also requires calculation of recovery time constants corrected for the diffusion of buffer between cell and patch pipette. The corrected time constant, τ\(_{\text{c}}\), was determined from Eq. 16 of Neher and Augustine (1992). The average time constant for fura-2 loading was 254 ± 29 s in 5K cells and 176 ± 23 s in 25K cells (means ± SE). To determine endogenous Ca\(^{2+}\) buffer capacity (K\(_{\text{B}}\)), τ\(_{\text{c}}\) was plotted versus K\(_{\text{B}}\) for the 5K and 25K populations (separately), and the points were fitted with linear regressions. The negative x intercept of such plots is 1 + K\(_{\text{B}}\) and the y intercept indicates the recovery time constant for the cell population in the absence of fura-2.

The ‘binding’ method (method 2 of Neher and Augustine 1992; see also Müller et al. 1993) determines the fraction of entering Ca that binds fura-2 by measuring the amplitudes of fluorescence changes that occur in response to Ca entry evoked by depolarization of fura-2-loaded cells. This was achieved by calculating the ratio (f) of the change in F\(_{380}\), to the time integral of I\(_{\text{Ca}}\) evoked by a depolarizing voltage-clamp step (f = ΔF\(_{380}\)/I\(_{\text{Ca}}\) dt) (Eq. 24, Neher and Augustine 1992). Plots of f versus K\(_{\text{B}}\) were fitted to the equation: f = k\(_{\text{B}}\) + K\(_{\text{B}}\) (1 + K\(_{\text{B}}\) + K\(_{\text{B}}\)) (Eq. 5, Müller et al. 1993) to yield k\(_{\text{B}}\), a proportionality constant, and K\(_{\text{B}}\), the endogenous Ca\(^{2+}\) buffer capacity. When plotted as a double reciprocal plot and fitted with a linear regression, the negative x intercept yields the value 1/(1 + K\(_{\text{B}}\)).

**Curve fitting**

The amplitudes and decay rates of [Ca\(^{2+}\)], transients were determined with PeakFit (version 3.11; Jandel Scientific, San Rafael, CA). Data were fit with a mono-exponential function, A\(_{\text{0}}\) * exp[−t/τ], where A\(_{\text{0}}\) is the initial amplitude, t is time, and τ is the time for the transient to decay by 1/e. Fits were accepted if r\(^2\) was ≥0.9. The time integrals of [Ca\(^{2+}\)], transients were determined for the period from transient onset to recovery to baseline (estimated from fits of the recovery phase) and were calculated with Lotus 123 (release 3.1). Linear regression fits and nonlinear curve fits were performed in SigmaPlot (version 5.0, for DOS; Jandel Scientific). The ranges of data to be fit
with linear regressions were selected by eye. All accepted fits had $R > 0.86$.

Statistics

All means are presented ±SE. The program Instat (GraphPAD Software, San Diego, CA) was used for most analyses. Large data sets were analyzed with ABstat (version 6, Anderson-Bell, Parker, CO). The significance of differences was determined by means of two-tailed parametric (paired or unpaired $t$-tests) or nonparametric tests (paired Wilcoxon signed rank test or unpaired Mann-Whitney 2 sample test) as appropriate.

To estimate the significance of correlations, the $t$-statistic was calculated from the correlation coefficient and the number of data points (Bradly et al., 1994), and $P$ values were then obtained from a two-tailed $t$-distribution table (Zar 1984). The linear fits of population data from 5K and 25K neurons were tested for significance of difference using two methods. The first method compares the slopes of the linear regressions of the two populations, testing for difference between the two population regression coefficients. The second method compares the two correlation coefficients (Zar 1984).

Solutions

Standard external solution (SES), used for $[\text{Ca}^{2+}]_i$ and AP measurements, consisted of Hank’s balanced salt solution (in mM) 137 NaCl, 5.4 KCl, 0.44 KH$_2$PO$_4$, and 0.34 Na$_2$HPO$_4$ supplemented with (in mM) 3.0 CaCl$_2$, 10 glucose, and 5 HEPES-Na-HEPES (pH 7.35). 25K SES consisted of SES adjusted to 25 mM KCl. For $I_{Ca}$ measurements, external solution contained (in mM) 100 TEA-Cl, 3 CaCl$_2$, 10 glucose, 50 sucrose, 8.5 TEA-OH, and 40 HEPES plus 3 μM tetродotoxin (pH 7.4). Recording pipettes used for whole cell current-clamp recording contained (in mM) 150 KCl, 2 MgCl$_2$, 1 EGTA, 1 ATP, 1 GTP, and 10 HEPES (pH 7.3). Pipettes used for perforated-patch current-clamp recordings contained (in mM) 130 K-gluconate, 20 KCl, 5 KOH, 5 MgCl$_2$, 10 glucose, and 10 HEPES (pH 7.3). Nystatin (20% wt/vol; 0.4% final DMSO concentration) was suspended in the pipette solution before use. Pipettes for perforated patch recordings of $I_{Ca}$ contained (in mM) 55 CsCl, 75 Cs$_2$SO$_4$, 7 MgCl$_2$, and 5 CsOH (pH 7.35). For whole cell $I_{Ca}$ recordings, pipettes contained (in mM) 100 CsCl, 15 CsOH, 2 MgCl$_2$, 22 sucrose, 1 ATP, 1 GTP, 5 bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), and 40 HEPES (pH 7.35). Fura-2 pentapotassium salt (500 μM) replaced BAPTA in this cesium-based internal solution for fura-2-loading experiments. All solutions contained 28 μM phenol red for pH indication.

Solution changes

Solution changes were made with a linear microcapillary array controlled by a hydraulic manipulator. The time for changes in the solution superfusing a cell was ~1.5 s. Positive pressure was supplied by a syringe pump operating at low flow rates (typically, 0.4–4.0 ml/h). The array was positioned so that the cells were exposed to flowing solution throughout the experiment.

Chemicals

All reagents were purchased from Sigma (St. Louis, MO) except for the following: 2,5-di-tert-butyl-hydroquinone, anhydrous dimethylsulfoxide (Aldrich, Milwaukee, WI); ryanodine (Calbiochem, La Jolla, CA); BAPTA, calcium calibration buffer kit II, fura-2 pentapotassium salt, fura-2 AM, Pluronic F-127 (Molecular Probes); and nitrendipine (Research Biochemicals, Natick, MA).

RESULTS

Action potentials, Ca currents, and $[\text{Ca}^{2+}]_i$ transients

Franklin et al. (1992) showed that chronic depolarization of myenteric neurons reduces the density of pharmacologically isolated Ca channel currents evoked by voltage-clamp steps. To determine the physiological consequences of these changes, we compared the waveforms and sensitivity of APs to Ca channel block in control neurons and in neurons that had been chronically depolarized for 3–5 days. Using either whole cell or perforated-patch recordings, we observed that the APs of chronically depolarized neurons were briefer (Fig. 1) and less sensitive to blockade of Ca currents by CdCl$_2$. The mean widths at half-height were 4.5 ± 0.2 and 5.0 ± 0.1 ms in 25K (n = 55) and 5K (n = 40) neurons, respectively (P < 0.04, 2-tailed unpaired $t$-test). CdCl$_2$ (100 μM), which completely blocks Ca currents in myenteric neurons (Franklin and Willard 1993), caused a 9% reduction in the amplitude and a 46% increase in the width of APs in 5K neurons but had no significant effect on the waveforms of 25K neurons. Chronic depolarization had no significant effect on the amplitudes of evoked APs or on their thresholds (data not shown). Thus the findings indicate that chronic depolarization reduced the contribution of Ca-dependent processes to AP waveform in myenteric neurons.

The decreased AP width associated with chronic depolarization may result in reduced Ca entry during electrical activity. Therefore Ca entry evoked by APs in 5K and 25K neurons was compared in two ways. First, Ca currents were evoked by voltage-clamp pulses designed to approximate the waveform of action potentials (APWs). An example is shown in Fig. 2. We found that identical APWs evoked smaller and briefer $I_{Ca}$ currents in 25K neurons than in 5K neurons. The mean densities of Ca influx evoked by APWs with a width at half-height of 5.0 ms (modeled after APs of 5K neurons) were 40.2 ± 7.6 fC/pF (n = 18) and 12.8 ± 2.8 fC/pF (n = 17) in 5K and 25K neurons, respectively (2-tailed $P < 0.0001$, unpaired Mann-Whitney 2 sample test). Decreasing the duration of the APW to 4.5 ms decreased Ca influx by <3% in 5K neurons and by approximately 14% in 25K neurons, suggesting that the ~70% reduction of Ca influx in 25K neurons (compared with 5K neurons)
neurons) is due mainly to lower Ca current density rather than to decreased AP width.

The second test of the hypothesis that APs evoke less Ca influx in 25K neurons was to compare the \([\text{Ca}^{2+}]_{i}\) transients evoked in 5K and 25K neurons by stimulation with extracellular field electrodes. Field stimulation allows measurement of \([\text{Ca}^{2+}]_{i}\) transients in a greater number of neurons per experiment and causes less disturbance of Ca homeostatic mechanisms than intracellular microelectrode recordings or whole cell recordings. The evoked \([\text{Ca}^{2+}]_{i}\) transients were attributed to Ca influx elicited by APs because they were blocked by removal of extracellular Ca or by addition of tetrodotoxin (Fig. 3). The transients had reproducible amplitudes and waveforms when sufficient time was allowed between stimulations for the transients to recover. They also closely resembled spontaneous transients that occasionally were observed. As expected from the decrease in Ca influx caused by chronic depolarization, single stimuli or brief trains of stimuli evoked smaller transients in 25K neurons than in 5K neurons (Fig. 4). During stimulus trains, \([\text{Ca}^{2+}]_{i}\) transients initially increased approximately linearly with the number of pulses and then increased at a slower rate. Cohen et al. (1997) observed a similar relationship between AP number and \([\text{Ca}^{2+}]_{i}\) transient amplitude in rabbit nodose neurons. Figure 4B shows that increasing the number of stimulus pulses caused a steeper rate of increase in \([\text{Ca}^{2+}]_{i}\) transients in 5K neurons than in 25K neurons. The altered relationship between AP number and transient amplitude suggests that chronic depolarization may have altered Ca handling by the neurons.

Indications that chronic depolarization altered components of Ca homeostasis in myenteric neurons came from further comparisons of the waveforms of \([\text{Ca}^{2+}]_{i}\) transients elicited in 5K neurons and 25K neurons. Figure 5, a comparison of the responses of populations of 5K and 25K neurons to standardized 8-pulse trains, shows that the \([\text{Ca}^{2+}]_{i}\) transients evoked in 25K neurons were smaller and
Chronic depolarization increases the ability of myenteric neurons to control \([\text{Ca}^{2+}]_i\).

Multiple elements of the intracellular Ca homeostatic system, including sequestration by organelles and efflux via plasma membrane mechanisms (reviewed in Carafoli 1987; Pozzan et al. 1994), designated here as slow Ca handling mechanisms, have been shown to reduce \([\text{Ca}^{2+}]_i\) on the time scale of seconds and to help regulate the waveform of \([\text{Ca}^{2+}]_i\) transients in a variety of neurons including bullfrog sympathetic neurons (Friel and Tsien 1992), rat DRG neurons (Werth et al. 1996), and Xenopus spinal neurons (Holliday et al. 1991). These relatively slow Ca clearance mechanisms combine with faster Ca binding mechanisms to shape \([\text{Ca}^{2+}]_i\) transients. To compare directly the Ca homeostatic capabilities of 5K and 25K neurons, we measured the \([\text{Ca}^{2+}]_i\) transients evoked by voltage-clamp steps. The time integrals of the Ca currents elicited by the voltage-clamp steps were used to calculate influx charge densities, which then were compared with the resultant \([\text{Ca}^{2+}]_i\) transients. Table 1 shows that the mean amplitude of \([\text{Ca}^{2+}]_i\) transients was significantly smaller in 25K neurons over a wide range of influx charge densities, indicating that chronic depolarization increased fast Ca handling capabilities in the myenteric neurons.

To test whether the increases in \([\text{Ca}^{2+}]_i\), due to larger Ca influx were activating additional Ca handling components (e.g., Stuenkel 1994; Thayer and Miller 1990), we examined the relationship of influx charge density to \([\text{Ca}^{2+}]_i\) transients. This relationship was linear at the lowest levels of Ca influx and then approached a limiting value, as illustrated in Fig. 6. Although the slopes of the linear phases were not significantly different in 5K (1.56 ± 0.17; \(n = 15\)) and 25K (1.29 ± 0.13; \(n = 15\)) neurons (2-tailed \(P = 0.21\), Mann-Whitney 2 sample test), there was a significant decrease in the limiting values from 968 ± 127 nM s \(s^{-1}\) (in 5K neurons) to 611 ± 96 nM s \(s^{-1}\) (in 25K neurons) (2-tailed \(P < 0.034\), unpaired Mann-Whitney 2 sample test). This suggests that the Ca handling mechanisms activated at higher levels of Ca influx may have been altered by chronic depolarization.

### Chronic depolarization increases fast Ca buffer capacity

Fast Ca buffers, such as calcium binding proteins, act to limit transient changes in \([\text{Ca}^{2+}]_i\). As such, we determined the fast Ca buffer capacities of 5K and 25K neurons by two methods that entail monitoring \([\text{Ca}^{2+}]_i\), transient waveforms during introduction of fura-2 into the neuronal cytoplasm from a patch pipet (Neher and Augustine 1992) (see METHODS). Both

![Graph](http://example.com/graph.png)

**FIG. 5.** \([\text{Ca}^{2+}]_i\) transients of chronically depolarized neurons are both smaller and faster. These cumulative probability curves show the distributions of decay time constants (A), amplitudes (B), and time integrals (C) of \([\text{Ca}^{2+}]_i\) transients evoked in 5K neurons (—) and 25K neurons (- - -) by trains of 8 pulses.

<table>
<thead>
<tr>
<th>Influx, (\text{fC/pF})</th>
<th>5K cells</th>
<th>25K cells</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(&lt;250)</td>
<td>22 ± 2.7 (28)</td>
<td>10 ± 0.9 (37)</td>
<td>0.001</td>
</tr>
<tr>
<td>250–500</td>
<td>39 ± 2.8 (13)</td>
<td>28 ± 1.7 (36)</td>
<td>0.001</td>
</tr>
<tr>
<td>500–1000</td>
<td>50 ± 2.9 (20)</td>
<td>31 ± 2.0 (26)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 1. Relationship of Ca influx to \([\text{Ca}^{2+}]_i\), transient

\([\text{Ca}^{2+}]_i\) transients were evoked by voltage-clamp pulses. Amplitudes of transients evoked in 5K and 25K neurons are means ± SE. Numbers in parentheses indicate number of cells tested. Significance of differences was assessed with unpaired Mann-Whitney two sample tests. 25K and 5K cells grown in medium that contains 25 or 5.4 mM KCl.
methods revealed that the fast buffering capacity of 25K neurons was increased significantly. Figures 7 and 8 illustrate raw and transformed data, respectively, from a 5K and a 25K neuron for analysis by the “binding” method, used to estimate endogenous Ca buffer capacity. By observing the consequences of loading individual cells with a concentration of fura-2 sufficient to alter evoked \([Ca^{2+}]_i\) transients, we estimated that the endogenous Ca buffer capacity increased from 172 \pm 44 (n = 9) in 5K neurons to 608 \pm 58 (n = 12) in 25K neurons (2-tailed \(P = 0.0005\), unpaired Mann-Whitney 2 sample test). By examining the effects of fura-2 on the recovery time constants of \([Ca^{2+}]_i\) transients in a group of 5K neurons and a group of 25K neurons (Fig. 9), we obtained a second estimate of the endogenous Ca buffer capacity, which increased from 132 in 5K neurons to 256 in 25K neurons. (Because this 2nd method of analysis compares pooled data for the 2 populations, there are no estimates of variance.) As was noted by Neher and Augustine (1992), estimates of buffer capacity based on recovery kinetics may be lower than those based on the binding method because of the presence of slow organellar Ca removal mechanisms. Analysis of the recovery time constants also allowed us to estimate that the “native” recovery time constants were 5.6 s in 5K neurons and 5.1 s in 25K neurons, supporting the conclusion from measurements of field stimulated \([Ca^{2+}]_i\) transients that chronic depolarization speeds the decay of the transients.

Chronic depolarization increases caffeine-releasable \(Ca^{2+}\) stores

Many neuronal cell types respond to caffeine application with an elevation of \([Ca^{2+}]_i\), indicating that they have caffeine-releasable Ca stores. Caffeine (10 mM) evoked \([Ca^{2+}]_i\) transients in the majority of 5K and 25K neurons, although the amplitudes and time courses of the responses were quite variable among different cells. Twenty-four of 30 5K cells and 18 of 19 25K cells had responses similar to those shown in Fig. 10. Initial responses to caffeine did not require extracellular Ca, but subsequent applications of caffeine in the absence of external Ca elicited little or no response (Fig. 10A). In contrast, in SES, the neurons responded to repeated caffeine applications. Figure 10A also shows that in Ca-free solution \([Ca^{2+}]_i\) fell below precaffeine levels after caffeine was washed out presumably due to depletion of intracellular Ca stores in the absence of external Ca. Ryanodine, which interacts with the caffeine-sensitive channels that mediate Ca-induced Ca release (CICR) (Kimball et al. 1996; McPherson et al. 1991), blocked the \([Ca^{2+}]_i\) responses to caffeine in a use-dependent manner and caused a slowly rising elevation of \([Ca^{2+}]_i\) (not shown). As summarized in Table 2, caffeine evoked larger and slightly more rapidly decaying \([Ca^{2+}]_i\) responses in 25K neurons than in 5K neurons. This appears not to be simply a consequence of the greater influx of Ca that occurred during culture in 25K neurons.
medium because caffeine evoked similar \([\text{Ca}^{2+}]_i\) transients regardless of whether it was applied in SES or in SES in which the [KCl] had been increased to 25 mM (Fig. 10, B and C).

Chronic depolarization changes the effects of ER-related \(\text{Ca}\) stores on electrically evoked \([\text{Ca}^{2+}]_i\) transients

Caffeine application increases \([\text{Ca}^{2+}]_i\) in most types of neurons. However, only in a subset of caffeine-responsive neurons does CICR contribute significantly to \([\text{Ca}^{2+}]_i\) responses

**FIG. 8.** Estimating endogenous \(\text{Ca}\) buffer capacity by the “binding” method. A and C: quantity \(f\) (the ratio of the change in \(F_{340}\) to \(F_{340} - F_{440}\)) is plotted as a function of \(\kappa_B\), the \(\text{Ca}\) binding capacity of the fura-2 diffusing into the 5K (A) and 25K (C) neurons that provided the records in Fig. 7. —, fit of the equation \(f = k'\kappa_B/(1 + \kappa_B + \kappa_S)\), where \(k\) is a proportionality constant and \(\kappa_S\) is the neuron’s endogenous fast \(\text{Ca}\) buffer capacity. B and D: double reciprocal plots of the data in A and C. —, linear regression fits. \(x\) intercepts of the fits occur at \(-1/(1 + \kappa_S)\). Estimates of \(\kappa_S\) were 102 and 571, respectively, for the 5K and 25K neurons shown here.

**FIG. 9.** Estimating endogenous \(\text{Ca}\) buffer capacity by the “kinetic” method. \(\tau^*\), which is the time constant of recovery of \([\text{Ca}^{2+}]_i\), corrected for diffusion of buffer between the neuron and the patch pipet, is plotted against \(\kappa_B\), the \(\text{Ca}\) binding capacity of fura-2. Slopes of the linear regression lines for data from 10 5K neurons (top, •) and from 12 25K neurons (bottom, ▲) are significantly different (\(P < 0.001\)). \(x\) intercepts of the lines \((1 + \kappa_S)\) yield estimates of endogenous \(\text{Ca}\) buffer capacity of 132 and 256 for the populations of 5K and 25K neurons, respectively.

**FIG. 10.** Caffeine-evoked \([\text{Ca}^{2+}]_i\) responses. A: caffeine (10 mM) was applied to a cluster of 5 5K neurons (monitored simultaneously) either in nominally \(\text{Ca}\)-free external solution or in SES. Second caffeine application in \(\text{Ca}\)-free solution elicited little change in \([\text{Ca}^{2+}]_i\), but after SES replaced the \(\text{Ca}\)-free solution, caffeine once again elicited a \([\text{Ca}^{2+}]_i\) transient. B and C: replacing SES with an external solution containing 25 mM KCl (25K) did not alter the amplitude or timecourse of the \([\text{Ca}^{2+}]_i\) transients evoked by application of caffeine to 5K (B) or 25K (C) neurons.

**Chronic depolarization changes the effects of ER-related \(\text{Ca}\) stores on electrically evoked \([\text{Ca}^{2+}]_i\) transients**

Caffeine application increases \([\text{Ca}^{2+}]_i\) in most types of neurons. However, only in a subset of caffeine-responsive neurons does CICR contribute significantly to \([\text{Ca}^{2+}]_i\) responses

**TABLE 2.** \([\text{Ca}^{2+}]_i\) responses evoked by 10 mM caffeine

<table>
<thead>
<tr>
<th></th>
<th>5K</th>
<th>25K</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude, nM</td>
<td>26.7 ± 2.7 (18)</td>
<td>61.2 ± 10.8 (15)</td>
<td>0.03</td>
</tr>
<tr>
<td>Decay time constant, s</td>
<td>23.4 ± 2.3 (16)</td>
<td>16 ± 3.6 (13)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

All cells had been grown in culture for 5 days and switched to standard external solution before caffeine application. Amplitudes and decay time constants of caffeine-evoked responses in 5K and 25K neurons are given as means ± SE. Numbers in parentheses indicate number of cells tested. Significance of differences was assessed with unpaired Mann-Whitney two sample tests.
depolarization reduced AP-evoked Ca release from caffeine-sensitive stores while it increased Ca uptake by caffeine-sensitive mechanisms.

In PC12 cells, release of Ca from caffeine-sensitive stores can be enhanced by release of Ca from inositol triphosphate (IP3)-sensitive stores (Reber et al. 1993). To test whether IP3-sensitive stores modulate [Ca2+], transients in myenteric neurons, we examined whether electrically evoked [Ca2+], transients were altered by thapsigargin (TG) and 2,5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ), which empty IP3-sensitive Ca stores by inhibiting ER Ca2+-ATPases (Kass et al. 1989; Thastrup et al. 1990). As illustrated in Fig. 11, B and C, TG elicited a slow increase in [Ca2+], in both 5K and 25K neurons that partially recovered during application. [Ca2+], responses to TG did not differ significantly in 5K and 25K neurons (data not shown). TG decreased the amplitudes of electrically evoked [Ca2+], transients in 5K neurons, and it slowed the decay of [Ca2+], transients in both cell types (Table 3). BHQ (10 μM) had effects similar to those of TG, although it usually elevated [Ca2+], for longer than did TG. Although the actions of TG and BHQ were similar to those of caffeine and ryanodine, the caffeine- and thapsigargin-sensitive Ca stores could be separated functionally because 10 mM caffeine evoked [Ca2+], responses in neurons that had already undergone an increase in [Ca2+], in response to 1 μM TG (not shown). Thus chronic depolarization may alter the possible role of IP3-sensitive Ca stores in Ca handling by myenteric neurons.

**DISCUSSION**

By using chronic depolarization as a tool to elevate [Ca2+], we have found that myenteric neurons marshal several responses, including decreased Ca influx during APs, reduced contribution of Ca from internal stores, increased fast Ca buffering, and increased ER clearance of cytoplasmic Ca, to restore [Ca2+], to an optimal level. These adaptive changes, incorporated in a schematic model in Fig. 12, demonstrate that myenteric neurons can adjust Ca homeostatic mechanisms to maintain [Ca2+], within a range optimal for normal functioning of Ca-dependent processes and to resist potentially lethal increases in [Ca2+].

**Decreased Ca influx during APs**

As would be predicted from the decreased Ca channel current, increased transient K current, and unchanged Na current observed by Franklin et al. (1992), 25K neurons had briefer APs and had lower densities of Ca influx when stimulated with voltage-clamp pulses that approximated APs. The decreased AP duration in 25K neurons appeared primarily to be due to increased K current density because total Ca channel blockade with Cd actually prolonged APs in 5K neurons and did not alter the duration of APs in 25K neurons. Chronic depolarization also decreases AP duration in chick sensory neurons, due to the appearance of a Ca-dependent, aminopyridine-sensitive, transient K current and to faster activation of a delayed rectifier K current (Yang and Zorumsky 1990). Comparison of the Ca influx evoked by voltage-clamp pulses that mimicked APs with durations characteristic of 5K and 25K neurons showed that the reduced Ca influx was attributable to reduced Ca current den-
[Ca\(^{2+}\)], transients were evoked by action potentials triggered by electrical field stimulation. All drugs used at 10 \(\mu\)M except caffeine (10 mM) and thapsigargin (1 \(\mu\)M). \(n\) (in parentheses) is sometimes smaller for decay rates than for amplitudes because analyses of decay rates were restricted to transients that could be fitted with single exponentials. Values (given as means \(\pm\) SE) for control and drug-treatment within each grouping and cell type are from the same cells. Different cells were used for the different treatments. BHQ, 2,5-di-(tert-butyl)-1,4-benzohydroquinone. *Paired Wilcoxon signed rank test. † Significant difference between fitted with single exponentials. Values (given as means

<table>
<thead>
<tr>
<th>Amplitude, nM</th>
<th>Time Constant, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>5K cells</td>
<td>25K cells</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>17.3 (\pm) 2.4 (16)</td>
</tr>
<tr>
<td>Percent change</td>
<td>-47</td>
</tr>
<tr>
<td>(P)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>50.7 (\pm) 6.0 (4)</td>
</tr>
<tr>
<td>Percent change</td>
<td>-47</td>
</tr>
<tr>
<td>(P)</td>
<td>&lt;0.05†</td>
</tr>
<tr>
<td>BHQ</td>
<td>39.4 (\pm) 3.4 (10)</td>
</tr>
<tr>
<td>Percent change</td>
<td>-71</td>
</tr>
<tr>
<td>(P)</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>28.3 (\pm) 1.8 (18)</td>
</tr>
<tr>
<td>Percent change</td>
<td>-48</td>
</tr>
<tr>
<td>(P)</td>
<td>&lt;0.01‡</td>
</tr>
</tbody>
</table>

Altered contributions of organellar Ca release and uptake to [Ca\(^{2+}\)], transients

The relationship between Ca influx and the amplitudes of [Ca\(^{2+}\)], transients was analyzed to assess the contributions of Ca release from intracellular stores, putatively labeled CICR, to [Ca\(^{2+}\)], transients evoked by electrical activity in myenteric neurons. At low levels of Ca influx, the amplitudes of [Ca\(^{2+}\)], transients increased linearly in both 5K and 25K neurons. Such linearity, which also is observed in rat DRG neurons (Thayer and Miller 1990), in bovine chromaffin cells (Neher and Augustine 1992), and in isolated rat neurohypophyseal nerve endings (Stuenkel 1994), suggests lack of a significant contribution to the transients by CICR under these conditions in myenteric neurons. In contrast, in cells in which CICR does make a significant contribution to [Ca\(^{2+}\)], transients, such as rat cerebellar Purkinje neurons (Llano et al. 1994) and rat DRG neurons (at higher levels of Ca influx) (Shmigol et al. 1995), a supralinear relationship has been reported. At higher levels of influx, the amplitudes of [Ca\(^{2+}\)], transients in myenteric neurons approached a limiting value (i.e., exhibit an infralinear relationship), as do rat sensory neurons in which Thayer and Miller (1990) attributed the infralinear relationship to Ca buffering actions of mitochondria. The lower limiting values for [Ca\(^{2+}\)], transient amplitude in 25K neurons suggest that the cellular mechanisms that handle large Ca loads have an increased capacity and/or that they are activated at lower [Ca\(^{2+}\)].

Analysis of the effects of caffeine, ryanodine, TG, and BHQ on basal [Ca\(^{2+}\)], and on the amplitude and time course of [Ca\(^{2+}\)], transients (as summarized in Table 3) permits us to draw conclusions about organellar stores and their contributions to [Ca\(^{2+}\)], transient waveform in myenteric neurons. The ability of these compounds to elevate [Ca\(^{2+}\)], in both 5K and 25K myenteric neurons indicates ER Ca stores the pharmacological properties of which are similar to those of many other cell types (Henzi and MacDermott 1992; Pozzan et al. 1994; Reber et al. 1993; Thayer and Miller 1990; Thayer et al. 1988). In addition, the lack of effects of caffeine, ryanodine, BHQ, and TG on the amplitude of AP-evoked [Ca\(^{2+}\)], transients in 25K neurons (Table 3) suggests that release of Ca from intracellular stores does not contribute significantly to [Ca\(^{2+}\)], transients in 25K neurons, whereas the ability of these compounds to reduce transients in 5K neurons suggests that Ca release does contribute to [Ca\(^{2+}\)], transients in 5K neurons. This latter conclusion seems to be in contradiction to the finding that the relationship of Ca influx to [Ca\(^{2+}\)], transient amplitude is infralinear in 5K neurons. However, similar findings have been described for largely CICR-mediated [Ca\(^{2+}\)], transients in rabbit vagal afferent neurons (Cohen et al. 1997). AP-induced [Ca\(^{2+}\)], transients in hippocampal neurons have a large contribution from Ca release from intracellular stores triggered by Ca influx as long as the extracellular Ca concentration is \(>50 \mu\)M (Jacobs and Meyer 1997). It is possible that the lack of effect of the pharmacological agents on [Ca\(^{2+}\)], transient amplitude in 25K neurons indicates a change in the sensitivity of the Ca release mechanism for Ca entry. This has yet to be tested.

The lack of effect of caffeine and ryanodine on [Ca\(^{2+}\)], transient amplitude in 25K neurons, in combination with the increase in caffeine-releasable Ca in these neurons, suggests
that chronic depolarization changes the role of caffeine-sensitive stores in 25K neurons, causing them to become more important for Ca sequestration than for Ca mobilization. It is possible that the sustained elevation of \([Ca^{2+}]_i\) caused by chronic depolarization may trigger a compensatory increase in the size of caffeine-sensitive stores, thereby enhancing the ability of myenteric neurons to control \([Ca^{2+}]_i\). Thus the factor (or factors) limiting the \([Ca^{2+}]_i\) transient amplitude in myenteric neurons remains to be described.

**Increased fast Ca buffer capacity**

Both methods of analysis revealed large (2- to 5-fold) increases in the endogenous Ca buffer capacity of 25K neurons. The estimates fall within the range previously estimated for other cell types (see Neher 1995 for review). However, as discussed by Zhou and Neher (1993), estimates of Ca binding capacity entail multiple assumptions and can be changed significantly by relatively small variation in measurements. In particular, it should be noted that estimates of binding capacity based on the analysis of recovery kinetics are less reliable (than estimates based on the binding method) because they attribute \([Ca^{2+}]_i\), transient decay kinetics exclusively to cytoplasmic Ca buffers, and we have evidence that ER \([Ca^{2+}]_i\), stores also contribute to these decay rates.

One plausible mechanism for increased fast Ca buffering capacity of 25K neurons would be increased expression of Ca binding proteins such as calbindin D28k. Vyas et al. (1994) reported that depolarization with 50 mM KCl or treatment with low concentrations of Ca ionophore induces calbindin D28k expression in PC12 cells. Transfection of GH3 cells with plasmids encoding calbindin D28k more than doubles their Ca buffering capacity (Lledo et al. 1992) and loading rat sensory neurons with calbindin D28k significantly reduces evoked \([Ca^{2+}]_i\), transients (Chard et al. 1993). Conversely, there is a significantly larger fast component in \([Ca^{2+}]_i\), transients in cerebellar Purkinje neurons from mice lacking calbindin D28k (Airaksinen et al. 1997). Calbindin D28k is found in about half of rat myenteric neurons in vivo (Buchan and Baimbridge 1988; Resibois et al. 1988), but the effect of \([Ca^{2+}]_i\), perturbations in myenteric neurons on the expression of calbindin D28k or other Ca binding proteins has not yet been elucidated.

**Increased \([Ca^{2+}]_i\), transient recovery**

The decay of \([Ca^{2+}]_i\), transients is faster in myenteric neurons that have undergone chronic depolarization, as measured during AP-induced transients and extrapolated from transients evoked by step depolarization. The extrapolated “native” recovery time constants, which represent the recovery in the absence of fura-2, were lower than recovery time constants measured in cells loaded with fura-2 AM, indicating that loading with the exogenous buffer affected the transients. Nevertheless, the lower recovery time constant in 25K neurons cannot be due to simple differences in dye loading in 5K and 25K neurons. Increasing the concentration of fura-2 should slow recovery rates (i.e., increase the recovery time constant) while decreasing \([Ca^{2+}]_i\), transient amplitude (e.g., Blumenfeld et al. 1992; Lledo et al. 1992). Also the reduced amplitudes of \([Ca^{2+}]_i\), transient in 25K neurons is not responsible for the faster recovery because transient amplitude did not affect the recovery rates in either 5K or 25K neurons. We therefore surmise that the faster decay of transients in 25K neurons is due to a combination of increased Ca sequestration by internal...
stores (as supported by the effects of thapsigargin and caffeine on time constants) and increased Ca efflux by, as yet, unidentified mechanisms. The determination of the mechanisms responsible for the faster $[\text{Ca}^{2+}]$, transient decay will be of great importance for understanding the adaptation of Ca homeostatic mechanisms to Ca perturbations.

**Ca-dependent changes in electrical properties**

The changes in Ca handling capabilities caused by chronic depolarization of myenteric neurons may be a consequence of the long-term elevation of $[\text{Ca}^{2+}]$, through a dihydropyridine-sensitive mechanism as has been determined for the sustained component of myenteric neuronal Ca current (Franklin et al. 1992). Activity-dependent increases in $[\text{Ca}^{2+}]$, that last hours to days can cause changes in the ionic conductances of other neurons, including cultured lobster stomatogastric ganglion (STG) neurons (Turrigiano et al. 1994) and crayfish motoneurons (Hong and Lennicka 1995). Importantly, in crayfish motoneurons, the activity-dependent decrease of Ca current density is accompanied by an increase in Ca clearance capability, resulting in faster decay of $[\text{Ca}^{2+}]$, transients (Lennicka et al. 1998). Modeling studies have shown that activity-dependent changes in maximal membrane conductances, regulated through Ca-dependent pathways, can alter dramatically the ability of STG neurons to generate patterned electrical activity (LeMasson et al. 1993; Liu et al. 1998). In guinea pig myenteric neurons, Ca-dependent K conductances can exert strong influence on firing patterns (reviewed in Wood 1989). Thus in addition to altering Ca homeostasis, changes in $[\text{Ca}^{2+}]$, induced by chronic depolarization also could alter the activity and/or expression of Ca-dependent conductances of myenteric neurons and thereby alter the patterns of APs they fire. It will be interesting to test whether altered patterns of action potential activity can induce Ca-dependent changes in Ca homeostasis.

**Implications of the upregulation of Ca homeostasis by chronic depolarization**

Nishi and Willard (1985) originally added depolarizing concentrations of KCl to culture medium to enhance long-term growth and survival of rat myenteric neurons in culture. The findings reported here support the hypothesis that chronic depolarization enhances neuronal survival by increasing their ability to control $[\text{Ca}^{2+}]$. The hypothesis that increased Ca handling capabilities will enhance neuronal survival under adverse conditions is consistent with several previous findings. Chronic depolarization decreases N-methyl-D-aspartate (NMDA)-induced $[\text{Ca}^{2+}]$, responses of mouse spinal neurons (Tymianski et al. 1994) and rat cerebellar granule cells (Pearson et al. 1992) and thereby decreases the excitotoxic effects of NMDA receptor stimulation. Chronically depolarized cerebellar granule cells are also resistant to apoptosis induced by a transforming growth factor, TGF-β (De Luca et al. 1996). Furthermore the ability of several neurotrophic factors and cytokines to reduce the elevation of $[\text{Ca}^{2+}]$, caused by metabolic insults suggests that stabilization of Ca homeostasis is a mechanism by which these agents reduce neuronal vulnerability (Cheng and Mattson 1991; Cheng et al. 1994). The relationship between Ca homeostasis and neuronal survival also is suggested by recent findings that Ca homeostatic mechanisms are subject to change with age in vivo in rat basal forebrain neurons and rat hippocampal neurons (Hartmann et al. 1996; Murchison and Griffith 1998) and may be correlated with the survival of cultured fetal rat hippocampal neurons (Porter et al. 1997). Future experiments should be directed toward discovering mechanisms by which elevation of $[\text{Ca}^{2+}]$, causes alterations in neuronal Ca homeostasis.

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