Ca\textsuperscript{2+}-dependent, stimulus-specific modulation of the plasma membrane 
Ca\textsuperscript{2+} pump in hippocampal neurons

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Running title: Ca\textsuperscript{2+}-dependent, stimulus-specific PMCA modulation in neurons

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Abstract

The plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) plays a major role in restoring Ca\textsuperscript{2+} to basal levels following transient elevation by neuronal activity. Here, we examined the effects of various stimuli that increase [Ca\textsuperscript{2+}]\textsubscript{i} on PMCA-mediated Ca\textsuperscript{2+} clearance from hippocampal neurons. We used indo-1 based microfluorimetry in the presence of cyclopiazonic acid to study the rate of PMCA-mediated recovery of Ca\textsuperscript{2+} elevated by a brief train of action potentials. [Ca\textsuperscript{2+}]\textsubscript{i} recovery was described by an exponential decay and the time constant provided an index of PMCA-mediated Ca\textsuperscript{2+} clearance. PMCA function was assessed before and for at least 60 min following a 10 minute priming stimulus of either 100 \textmu M NMDA, 0.1 mM Mg\textsuperscript{2+} (reduced extracellular Mg\textsuperscript{2+} induces intense excitatory synaptic activity), 30 mM K\textsuperscript{+}, or control buffer. Recovery kinetics slowed progressively following priming with NMDA or 0.1 mM Mg\textsuperscript{2+}; in contrast, Ca\textsuperscript{2+} clearance initially accelerated and then slowly returned to initial rates following priming with 30 mM K\textsuperscript{+}-induced depolarization. Treatment with 10 \textmu M calpeptin, an inhibitor of the Ca\textsuperscript{2+} activated protease calpain, prevented the slowing of kinetics observed following treatment with NMDA, but had no affect on the recovery kinetics of control cells. Calpeptin also blocked the rapid acceleration of Ca\textsuperscript{2+} clearance following depolarization. In calpeptin-treated cells, 0.1 mM Mg\textsuperscript{2+} induced a graded acceleration of Ca\textsuperscript{2+} clearance. Thus, in spite of producing comparable increases in [Ca\textsuperscript{2+}]\textsubscript{i}, activation of NMDA receptors, depolarization-induced activation of voltage-gated Ca\textsuperscript{2+} channels and excitatory synaptic activity each uniquely affected Ca\textsuperscript{2+} clearance kinetics mediated by the PMCA.
Introduction

The plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) is an integral part of the Ca\textsuperscript{2+} regulatory system in neurons (Thayer et al. 2002) and plays a prominent role in returning [Ca\textsuperscript{2+}]	extsubscript{i} to basal levels following moderate stimuli (Benham et al. 1992; Werth et al. 1996). These Ca\textsuperscript{2+} pumps hydrolyze ATP to enable the translocation of Ca\textsuperscript{2+} up the steep gradient across the plasma membrane (Carafoli and Brini 2000). PMCA diversity results from the alternative splicing of 4 primary transcripts (Strehler and Zacharias 2001) to produce various pump isoforms differing in their distribution within the brain (Stauffer et al. 1995), subcellular localization (DeMarco and Strehler 2001), activity (Enyedi et al. 1994) and modulation (Enyedi et al. 1996; Usachev et al. 2002). Plasma membrane Ca\textsuperscript{2+} pumps regulate a variety of Ca\textsuperscript{2+} signaling processes including neurotransmitter release (Empson et al. 2007; Zenisek and Matthews 2000) and excitability (Usachev et al. 2002). Changes in PMCA function accompany aging (Murchison and Griffith 1998), free radical damage (Zaidi et al. 2003) and excitotoxicity (Pottorf et al. 2006).

Ca\textsuperscript{2+} is not only transported by the PMCA but also regulates its activity. The carboxyl terminal of most PMCA isoforms contains an autoinhibitory domain that, in the absence of Ca\textsuperscript{2+}-calmodulin, inhibits Ca\textsuperscript{2+} pump activity by intramolecular binding to a site near the ATP catalytic site (Enyedi et al. 1991). Calmodulin in the presence of Ca\textsuperscript{2+} binds to the autoinhibitory domain removing inhibition and thus, stimulates Ca\textsuperscript{2+} pump activity (Carafoli 1994). The PMCA is also modulated by the Ca\textsuperscript{2+} activated protease calpain. Calpain-mediated cleavage of the carboxyl terminus autoinhibitory domain results in PMCA activation (James et al. 1989). Alternatively, glutamate-induced calpain
activation results in loss of PMCA activity possibly due to internalization of the proteolyzed PMCA (Pottorf et al. 2006).

In this report, we describe the effects of various stimuli that elevate [Ca\textsuperscript{2+}]\textsubscript{i} on PMCA function. We found that PMCA-mediated Ca\textsuperscript{2+} clearance was affected in a Ca\textsuperscript{2+} source specific manner and that calpain activation was a major contributor to PMCA modulation.

Methods

Cell culture. Rat hippocampal neurons were grown in primary culture as described previously (Wang et al. 1994) with minor modifications. Fetuses were removed on embryonic day 17 from maternal rats, anesthetized with CO\textsubscript{2}, and sacrificed by decapitation under a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Hippocampi were dissected and placed in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free HEPES buffered Hanks’ salt solution (HHSS), pH 7.45. HHSS was composed of the following (in mM): HEPES 20, NaCl 137, CaCl\textsubscript{2} 1.3, MgSO\textsubscript{4} 0.4, MgCl\textsubscript{2} 0.5, KCl 5.0, KH\textsubscript{2}PO\textsubscript{4} 0.4, Na\textsubscript{2}HPO\textsubscript{4} 0.6, NaHCO\textsubscript{3} 3.0, and glucose 5.6. Cells were dissociated by trituration through a 5 ml pipette and a series of flame-narrowed Pasteur pipettes. Cells were pelleted and re-suspended in Dulbecco’s Modified Eagle’s Medium (DMEM) without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). Dissociated cells then were plated at a density of 10,000-15,000 cells/well onto 25-mm-round cover glasses that had been coated with poly-D-lysine (0.1 mg/ml) and washed with H\textsubscript{2}O. Neurons were grown in a humidified atmosphere of 10% CO\textsubscript{2} and 90% air (pH 7.4) at 37 °C, and fed at
days 1 and 6 by exchange of 75% of the media with DMEM supplemented with 10% horse serum and penicillin/streptomycin. Cells used in these experiments were cultured without mitotic inhibitors for a minimum of 10 days and a maximum of 15 days.

**Microfluorometric recording of \([Ca^{2+}]_i\).** \([Ca^{2+}]_i\) was recorded from cultured rat hippocampal neurons using indo-1-based microfluorometry (Gryniewicz et al. 1985). Cells were loaded with 2 \(\mu\)M indo-1 acetoxyethyl ester (AM) in HHSS containing 0.5% bovine serum albumin at 37°C for 30 min. Cells were rinsed in HHSS and the indicator allowed to de-esterify for 10 min prior to starting the experiment. Coverslips with loaded, washed cells were mounted in a flow-through chamber (10 s solution exchange) equipped with platinum electrodes (Thayer et al. 1988). The chamber was mounted on an inverted epi-fluorescence microscope, and cells localized by phase-contrast illumination with a 70x objective (NA 1.15). Action potentials were evoked by electric field stimulation as described previously (Piser et al. 1994). For high extracellular K\(^+\) concentration ([K\(^+\)]_o)-induced depolarization, K\(^+\) was substituted for Na\(^+\) reciprocally. Experiments were performed at room temperature (22°C). The dye was excited at 350 nm (10 nm bandpass), and emission was detected at 405 (20) and 490 (20) nm. Fluorescence was monitored by a pair of photomultiplier tubes (Thorn, EMI, Fairfield, NJ) operating in photon-counting mode. The output signals were then passed through a frequency to voltage converter and digitized using a Digidata 1322a A/D Converter (MDS Analytical Technologies, Toronto, CA). Data were stored and analyzed on a personal computer.

Fluorescence changes were converted to \([Ca^{2+}]_i\) by using the formula \([Ca^{2+}]_i = K_d \beta (R - R_{min})/(R_{max} - R)\), where R is 405/490 nm fluorescence ratio (Gryniewicz et al.
1985). The dissociation constant ($K_d$) for indo-1 was 250 nM and $\beta$ was the ratio of fluorescence emitted at 490 nm and measured in the absence and presence of $\text{Ca}^{2+}$. $R_{\text{min}}$, $R_{\text{max}}$, and $\beta$ were determined by bathing intact cells in 2 $\mu$M ionomycin in $\text{Ca}^{2+}$-free buffer (1 mM EGTA) and saturating $\text{Ca}^{2+}$ (5 mM $\text{Ca}^{2+}$). Values for $R_{\text{min}}$, $R_{\text{max}}$, and $\beta$ were 0.25, 2.3 and 3.5, respectively. After completion of each experiment, cells were wiped from the microscope field using a cotton-tipped applicator. Background light levels were determined at each wavelength (approximately 5% of cell counts) and subtracted prior to calculating ratios.

**Statistical Analysis.** The recovery of intracellular calcium to resting levels was quantified by fitting a monoexponential decay function using a non-linear, least-squares curve fitting algorithm (Origin 6.1 software) (Usachev et al. 2006). Statistical significance was determined by univariate ANOVA with Tukeys post-hoc test or by Student’s t-test as indicated, an acceptable level of significance was $\alpha = 0.05$ (SPSS 14.0 software).

**Reagents.** Indo-1, DMEM, fetal bovine serum, and horse serum were obtained from Invitrogen (Carlsbad, CA). Calpeptin was purchased from Calbiochem (San Diego, CA) and all other reagents were obtained from Sigma (St. Louis, MO).
RESULTS

A method to study PMCA recovery kinetics in hippocampal neurons.

The clearance of small (<400 nM) increases in [Ca\(^{2+}\)]\(_i\) from the neuronal cytoplasm is primarily accomplished by ATPases that pump Ca\(^{2+}\) across the plasmalemma and into the endoplasmic reticulum (Usachev et al. 2006). We evoked brief trains of action potentials in indo-1AM loaded hippocampal neurons by electrical field stimulation (40-90V; 4-8 Hz, 4 s) to elicit a rapid increase in [Ca\(^{2+}\)]\(_i\) (Fig. 1). The [Ca\(^{2+}\)]\(_i\) returned to basal levels by a process well fit by a mono-exponential equation with a mean time constant (\(\tau\)) of 2.2 ± 0.6s (n=27) (Fig. 1B). Application of cyclopiazonic acid (5 µM, CPA) to block the sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), produced a transient rise in basal [Ca\(^{2+}\)]\(_i\) and a slowing of the [Ca\(^{2+}\)]\(_i\) recovery kinetics (\(\tau = 4.5 ± 1.8\) s, n=80). We have shown previously that in the presence of CPA, recovery from action potential-induced [Ca\(^{2+}\)]\(_i\) increases of less than 400 nM is not significantly influenced by mitochondrial Ca\(^{2+}\) uptake or Na\(^+\)/Ca\(^{2+}\) exchange. Under these conditions, recovery is predominantly mediated by the PMCA (Pottorf et al. 2006).

We used recovery from these small action-potential-induced [Ca\(^{2+}\)]\(_i\) increases as tests of PMCA function in hippocampal neurons and measured \(\tau\) for at least an hour following challenges with various stimuli. Figure 1 shows an example of this type of experiment. Every 4 min the cell received electrical field stimulation to evoke a brief train of action potentials to elicit a reproducible increase in [Ca\(^{2+}\)]\(_i\). Initial recovery kinetics (\(\tau_0\)) were defined as the mean of the two \(\tau\) values calculated for [Ca\(^{2+}\)]\(_i\) transients recorded after the addition of CPA to the superfusate (b and c in Fig. 1 A). Two minutes after the second control response (t = 2 min) [Ca\(^{2+}\)]\(_i\) was elevated by applying a test
stimulus. For the experiment shown in Figure 1 the cell was bathed in HHSS in which the extracellular Mg$^{2+}$ concentration ([Mg$^{2+}$]$_o$) was reduced from 0.9 to 0.1 mM. Reduced [Mg$^{2+}$]$_o$ induces an epileptic pattern of activity in the synaptic network that forms in hippocampal cultures and is manifest as a series of [Ca$^{2+}$]$_i$ spikes (McLeod et al. 1998). Following a 10 min exposure to 0.1 mM [Mg$^{2+}$]$_o$, PMCA-mediated recovery kinetics were monitored for over 1 hr. The reduced [Mg$^{2+}$]$_o$ initiated a graded slowing of PMCA-mediated [Ca$^{2+}$]$_i$ clearance (Fig. 1C).

In control recordings, no stimulus was applied during the priming period (Fig. 2A) and thus, [Ca$^{2+}$]$_i$ remained at basal levels. PMCA-mediated [Ca$^{2+}$]$_i$ recovery kinetics were stable over the time course of these experiments (Fig. 2B). The slight acceleration suggested by the negative slope of a plot of $\tau/\tau_0$ versus time was not statistically significant (Fig. 2C)(n=5). The Ca$^{2+}$ activated protease calpain can accelerate or impair PMCA activity depending on conditions (James et al. 1989; Pottorf et al. 2006). However, under control conditions the calpain inhibitor calpeptin (10 $\mu$M) did not affect PMCA function (Fig. 2C)(n=6). Thus, in the absence of a priming stimulus, calpain was not activated and PMCA-mediated [Ca$^{2+}$]$_i$ recovery kinetics were stable for the duration of the experiment.

**Stimulus-induced changes in PMCA recovery kinetics**

Treating hippocampal neurons with 0.1 mM [Mg$^{2+}$]$_o$ as described in Figure 1 induced an intense pattern of excitatory synaptic activity that results in a series of [Ca$^{2+}$]$_i$ spikes (Fig. 3A). Reducing [Mg$^{2+}$]$_o$ induces paroxysmal excitatory bursts analogous to epileptic activity (Rose et al. 1990). Following 10 min exposure to 0.1 mM [Mg$^{2+}$]$_o$, PMCA mediated recovery kinetics displayed a graded slowing (Fig. 1C and Fig. 3B). $\tau$
increased at a rate of 0.006 min\(^{-1}\) and was significantly greater than control cells (p<0.05). In cells treated with calpeptin for at least 20 minutes prior to the stimulus and throughout the recording, the 0.1 mM \([\text{Mg}^2+]_o\) priming stimulus initiated a graded acceleration of PMCA-mediated \([\text{Ca}^{2+}]_i\) clearance (Fig. 3B). Thus, in calpeptin-treated cells 0.1 mM \([\text{Mg}^2+]_o\) induced an increase in \(\text{Ca}^{2+}\) clearance rate of 0.0015 min\(^{-1}\) which was significantly faster than control cells (p<0.01). Calpeptin did not affect 0.1 mM \([\text{Mg}^2+]_o\)-induced \([\text{Ca}^{2+}]_i\) spiking activity (Fig. 3C). The number of \([\text{Ca}^{2+}]_i\) spikes during the 10 min treatment was 45 ± 17 in untreated cells and 36 ± 10 in the presence of calpeptin, and the difference between them was not significant \((t_{7} = -1.36)\). The mean spike amplitude in untreated and treated cells was 469 ± 216 nM and 495 ± 218 nM, respectively, and the difference between them was not significant \((t_{325} = 1.1)\). Thus, in the presence of calpeptin the same \([\text{Ca}^{2+}]_i\) increase that caused a calpain-mediated slowing of \(\text{Ca}^{2+}\) clearance, now produced an acceleration of PMCA activity (Fig. 3B and D)(p<0.01). The source of \(\text{Ca}^{2+}\) during 0.1 mM \([\text{Mg}^2+]_o\)-induced \([\text{Ca}^{2+}]_i\) spiking is from both voltage-gated \(\text{Ca}^{2+}\) channels and NMDA receptors (McLeod et al. 1998). We next examined the effects of stimulating these channels individually on \(\text{Ca}^{2+}\) clearance rate.

NMDA (100 \(\mu\)M) induced a large \([\text{Ca}^{2+}]_i\) increase that peaked at 777 ± 208 nM and declined to 337 ± 113 nM by the end of the 10 min priming stimulus (Fig. 4A). This stimulus produced a graded slowing in PMCA-mediated recovery kinetics (Fig. 4B, E). \(\tau\) increased at a rate of 0.007 min\(^{-1}\) and was significantly greater than control cells (p<0.01). In contrast, NMDA did not significantly affect PMCA function in the presence of calpeptin (Fig. 4D and E). Calpeptin did not affect the NMDA-induced \([\text{Ca}^{2+}]_i\) increase (Fig. 4C). The \([\text{Ca}^{2+}]_i\) peaked at 934 ± 160 nM and declined to 466 ± 183 nM by
10 min, similar to responses recorded from untreated cells ($t_8 = 1.3, 1.4$, respectively). Thus, 10 min treatment with NMDA induced a calpain-mediated slowing of PMCA activity.

Activation of VGCCs by elevating the extracellular K$^+$ concentration ([K$^+$]$_o$) to 30 mM produced a sustained elevation in [Ca$^{2+}$]$_i$ that peaked at 1463 ± 532 nM (Fig. 5A). In contrast to the other stimuli examined here, activation of VGCCs produced a rapid acceleration of Ca$^{2+}$ clearance that gradually returned to the original rate (Fig. 5B and E). The y-intercept extrapolated from the data in Fig. 5E was 0.5 and the recovery time constant ($\tau/\tau_o$) increased at a rate of 0.005 min$^{-1}$. In the presence of calpeptin (10 µM) the 30 mM [K$^+$]$_o$ stimulus did not significantly affect recovery kinetics, although an accelerating trend in PMCA activity was apparent (Fig. 5E). Calpeptin did not significantly affect the depolarization-induced [Ca$^{2+}$]$_i$ increase (peak [Ca$^{2+}$]$_i$ = 1658 ± 427 nM; $t_9 = 0.66$ (Fig. 5C)). Thus, depolarization-induced Ca$^{2+}$ influx produced an initial calpain-dependent acceleration of Ca$^{2+}$ clearance that gradually returned to the initial rate.

Because the [Ca$^{2+}$]$_i$ increases induced by the NMDA and 30 mM K$^+$ priming stimuli were comparable, but subsequent changes in PMCA-mediated Ca$^{2+}$ clearance were different, we examined the effects of priming with different concentrations of NMDA. The [Ca$^{2+}$]$_i$ increases evoked by 10, 30 and 100 µM NMDA were graded with peak values ranging from 436 to 1896 nM. There was no correlation between the amplitude of the Ca$^{2+}$ increase and subsequent changes in PMCA-mediated [Ca$^{2+}$]$_i$ recovery kinetics ($r^2<0.01$; n=19). Thus, global changes in [Ca$^{2+}$]$_i$ did not predict changes in PMCA function. Perhaps depolarization and NMDA create stimulus
dependent Ca\textsuperscript{2+} microdomains that are not resolved by the whole-cell measurements used here.

**Stimulus-induced time-dependent changes in PMCA function are dependent on the Ca\textsuperscript{2+} source**

In order to compare the changes in PMCA function resulting from each of the stimuli, we analyzed our entire data set. First we compared the effects of each stimulus on the change in \(\tau\) for both untreated (filled bars) and calpeptin (open bars) conditions (Fig. 6A). \(\tau\) did not significantly change under control conditions or following depolarization (30 mM K\textsuperscript{+}). However, activation of NMDA receptors either directly or via synaptic activity (0.1 mM [Mg\textsuperscript{2+}]\textsubscript{o}) produced a long-term slowing of PMCA function (p<0.01 and p<0.05, respectively). Calpain mediated this slowing as indicated by significantly reduced mean \(\tau\) values for cells treated with NMDA (p<0.001) and 0.1 mM [Mg\textsuperscript{2+}]\textsubscript{o} (p<0.01) in the presence of calpeptin. We have previously described a glutamate-induced, calpain-mediated slowing of PMCA function that correlated with internalization of the PMCA (Pottorf et al. 2006). However, we did not observe a detectable internalization of PMCA over the much shorter time course (1 versus 4 hr) studied here (data not shown).

The mean data show differences between test conditions but do not represent change over time. To assess this dynamic, a linear regression was performed on each series of time points recorded after the priming period and the slope and y-intercept of the experiments that significantly matched a linear fit (33 out of total of 41 experiments) are presented in Figures 6 B and C, respectively. Figures 2c, 3b, 4e and 5e show examples of
the recovery data for each priming condition and the fitted regression lines. The slope of the line reveals the rate of change of the PMCA-mediated recovery kinetics over the course of the experiment (Fig. 6b) and the projected initial rate of PMCA-mediated 

\[ [\text{Ca}^{2+}]_i \] clearance is provided by the y-intercept (Fig. 6c).

All three stimuli produced a graded slowing in PMCA function (Fig. 6B) and differed significantly from control (p<0.05). However, note that the Y-intercept derived from the plot of \( \tau/\tau_o \) versus time was significantly lower than control in 30 mM \([K^+]_o\) treated cells (Fig. 6C)(p<0.01). The change in the regression y-intercept (\( \tau/\tau_o \)) suggests that activation of VGCCs induced rapid changes in PMCA activity that occurred during the stimulus. The rapid depolarization-induced acceleration (Fig. 6C) as well as the gradual slowing that developed following all three stimuli were blocked by calpeptin (Fig. 6B). Thus, depolarization appears to activate calpain to produce a rapid stimulation of the PMCA followed by a more slowly developing inactivation. Only the slowly developing inactivation was observed following NMDA. Depolarization or intense synaptic activity (0.1 mM \([\text{Mg}^{2+}]_o\)) induced a graded acceleration of PMCA-mediated 

\[ [\text{Ca}^{2+}]_i \] clearance in the presence of calpeptin (p<0.01 relative to control).

**Discussion**

Following increases in \([\text{Ca}^{2+}]_i\), PMCA-mediated \( \text{Ca}^{2+} \) clearance underwent time and stimulus dependent changes in kinetics. Activation of NMDA receptors either directly or through excitatory synaptic activity induced a graded slowing of PMCA function that was mediated by calpain. In contrast, depolarization-induced activation of VGCCs induced an initial acceleration of PMCA activity followed by a graded slowing
that was also mediated by calpain. With calpain blocked, synaptically induced increases in \([\text{Ca}^{2+}]_i\) induced a graded acceleration in PMCA-mediated \(\text{Ca}^{2+}\) clearance. Thus, we resolved 3 \(\text{Ca}^{2+}\)-induced changes in PMCA function, a graded slowing, a rapid acceleration and a graded acceleration; the predominant effect was determined by the specific source of \(\text{Ca}^{2+}\) and the presence of functional calpain.

The slowly developing loss of PMCA activity followed all three stimuli. Since the \([\text{Ca}^{2+}]_i\) was at basal levels during the progressive loss of PMCA function, the process was triggered by the stimulus-induced \([\text{Ca}^{2+}]_i\) increase but once started, continued in the absence of elevated \([\text{Ca}^{2+}]_i\). The block of this process by calpeptin suggests that the most parsimonious explanation for this sustained \(\text{Ca}^{2+}\) independent change in pump function is the autocatalytic activation of calpain. In the presence of a large, sustained increase in \([\text{Ca}^{2+}]_i\), calpain undergoes autoproteolysis that reduces the \([\text{Ca}^{2+}]_i\) required for activation (Goll et al. 2003). Large increases in \([\text{Ca}^{2+}]_i\) may also alter other aspects of calpain regulation such as inhibition by calpastatin. There are sites for calpain cleavage that are known to inactivate certain PMCA isoforms (Brown and Dean 2007). However, we do not know whether calpain acts directly on the PMCA or acts on one of its many other substrates to indirectly inhibit pump function (Goll et al. 2003; Wu and Lynch 2006). We have previously shown that the PMCA internalizes 4 hrs after starting a 30 min treatment with glutamate (Pottorf et al. 2006). The internalization required calpain activation and was accompanied by a loss of function. We were not able to detect PMCA internalization using the 10 min stimulus protocol employed in this study; whether this was due to insufficient resolution to detect small changes in PMCA distribution or whether pump inactivation precedes internalization is not clear. Both NMDA and low
[Mg$^{2+}$]$_o$ stimuli are potentially toxic, so the loss of PMCA activity following these treatments may contribute to the [Ca$^{2+}$]$_i$ dysregulation that is a hallmark of excitotoxicity (Randall and Thayer 1992). The depolarization-induced slowing of PMCA-mediated Ca$^{2+}$ clearance follows more rapid acceleration and thus, may be a homeostatic process.

The biphasic changes in PMCA function induced by 30 mM [K$^+$]$_o$ suggest a two step process. The initial acceleration appears similar to the depolarization-induced stimulation of PMCA function described for sensory neurons (Pottorf and Thayer 2002) and fits well with a model of rapid Ca$^{2+}$-induced stimulation of PMCA activity that slowly inactivates due to the slow dissociation of calmodulin from certain PMCA isoforms (Caride et al. 2001). However, because calpeptin prevented the stimulation, our data are more consistent with the proteolytic activation of the PMCA by calpain-mediated cleavage of the inhibitory domain on the carboxy terminus (James et al. 1989). The return to basal activity may be via the same mechanism as the loss of function following NMDA. In this scenario calpain first activates the pump by cleavage of the carboxy terminus followed by a secondary inactivation mediated either by direct proteolysis of an inactivating site on the PMCA (Brown and Dean 2007) or via indirect mechanisms (Goll et al. 2003; Wu and Lynch 2006).

This conclusion raises the question as to why we do not see the initial activation of PMCA function in NMDA treated cells; after all, NMDA activated calpain too. Because the rate of PMCA inactivation following all three stimuli was similar (no statistical difference between the slopes in figure 6B), it seems implausible that the inactivation mechanism is more rapid following NMDA receptor activation. Perhaps the C-terminal calpain cleavage site is less accessible to calpain activated by NMDA.
receptor-mediated relative to VGCC-mediated Ca\(^{2+}\) influx. The susceptibility of certain calpain substrates to cleavage is regulated by phosphorylation. For example, phosphorylation of the NR2b subunit by the kinase fyn increases the susceptibility of the NMDA receptor to calpain-mediated cleavage (Wu et al. 2007). Calmodulin binding to the PMCA protects a C-terminal cleavage site by shielding it from calpain (Padanyi et al. 2003), establishing a precedent for modulation of PMCA susceptibility as a substrate. We speculate that the various stimuli differentially activate rapid, Ca\(^{2+}\)-dependent processes that modulate PMCA susceptibility to C-terminus cleavage by calpain. The differential localization of Ca\(^{2+}\)-sensitive signaling cascades at NMDA receptor versus VGCC by attachment to scaffolding proteins is well documented (Sattler et al. 1999; Weick et al. 2003).

The selective activation of a Ca\(^{2+}\) sensitive signaling cascade may also explain the slowly developing acceleration that follows exposure to low [Mg\(^{2+}\)]\(_o\) in the presence of calpeptin. The slowly developing nature of the increase in PMCA activity would seem to rule out direct activation by Ca\(^{2+}\)-calmodulin. Kinase activation would be a suitable mechanism in that PMCAs can be stimulated by phosphorylation (Penniston and Enyedi 1998; Usachev et al. 2002) and Ca\(^{2+}\)-induced auto-phosphorylation is known to produce sustained kinase activity in neurons (Lisman et al. 2002). Whatever the mechanism, it appears that Ca\(^{2+}\) influx via VGCCs is able to stimulate PMCA activity but not Ca\(^{2+}\) influx via NMDA receptors. Since the slowly developing acceleration is only apparent in the presence of calpeptin, its relevance to cell signaling is unclear. It appears to be dominated by the calpain-mediated slowing of pump function consistent with the idea that calpain inactivates the pump irreversibly. Perhaps under other conditions or
following different stimuli an acceleration of PMCA activity is more pronounced. Identifying these conditions and the mechanism are interesting future directions.

The large increase in \([\text{Ca}^{2+}]_i\) induced by the priming stimuli studied here would be expected to increase mitochondrial \(\text{Ca}^{2+}\) levels. A prolonged elevation in \([\text{Ca}^{2+}]_i\) due to mitochondrial \(\text{Ca}^{2+}\) buffering could not account for changes in PMCA function described here because the \([\text{Ca}^{2+}]_i\) was allowed to recover to basal levels before test stimuli were applied to determine PMCA kinetics. \(\text{Ca}^{2+}\)-induced, mitochondrial-mediated changes in cytoplasmic pH or ATP levels (Wang et al. 1994) could influence the ATP-dependent \(\text{Ca}^{2+}\)-\(\text{H}^+\) exchange mediated by the PMCA (Thomas 2008). However, \(\text{Ca}^{2+}\) taken up into the mitochondria following stimuli that evoke large \(\text{Ca}^{2+}\) loads is eventually removed from the mitochondrial matrix (Wang and Thayer 1996; Werth and Thayer 1994) which, would seem at odds with the idea that matrix \(\text{Ca}^{2+}\) levels produced the graded slowing in PMCA mediated \(\text{Ca}^{2+}\) clearance.

Changes in the rate of \(\text{Ca}^{2+}\) clearance modulate both physiological and pathological function. For example, \(\text{Ca}^{2+}\) clearance from dendritic spines is slowed following stimuli that induce long-term potentiation of synaptic transmission (Scheuss et al. 2006) and neurotoxic stimuli induce \(\text{Ca}^{2+}\) dysregulation that contributes to cell death (Mattson et al. 1992; Randall and Thayer 1992). Activation of protein kinase C in sensory neurons stimulated PMCA-mediated \(\text{Ca}^{2+}\) clearance resulting in an increase in excitability due to reduced activation of \(\text{Ca}^{2+}\) activated \(\text{K}^+\) channels (Usachev et al. 2002). Thus, it is important to understand how specific stimuli influence PMCA-mediated \(\text{Ca}^{2+}\) clearance. Clearly, stimuli that induced excitotoxicity such as NMDA and 0.1 mM Mg\(^{2+}\)-induced synaptic activity slowed \(\text{Ca}^{2+}\) clearance whereas depolarization, a stimulus that
triggers Ca\textsuperscript{2+} influx via VGCC and does not reduce survival, did not produce a net change in Ca\textsuperscript{2+} clearance rate. Transient decreases in Ca\textsuperscript{2+} clearance might modify Ca\textsuperscript{2+} signaling by lowering the stimulus threshold for initiating Ca\textsuperscript{2+} triggered events and by prolonging the duration of Ca\textsuperscript{2+} signals. Mutations in PMCA genes that reduce function are genetic modifiers that in the case of PMCA2 increase the risk for noise-induced, Ca\textsuperscript{2+}-dependent damage to the auditory system (Kozel et al. 2002).

Depolarization-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i} were larger than those induced by 100 µM NMDA but, did not produce a net change in Ca\textsuperscript{2+} clearance rate. We interpret this result to indicate that the source of Ca\textsuperscript{2+} determined the subsequent response. There is precedent for different Ca\textsuperscript{2+} sources activating distinct signaling cascades. For example, NMDA-evoked Ca\textsuperscript{2+} entry activates nitric oxide synthase (NOS) whereas a comparable depolarization-induced increase in cellular [Ca\textsuperscript{2+}]\textsubscript{i} did not. This selectivity resulted from the localization of NOS close to the mouth of the NMDA-gated ion channel such that it experienced a [Ca\textsuperscript{2+}]\textsubscript{i} increase much higher than the global increase that spread throughout the cytoplasm (Sattler et al. 1999; Weick et al. 2003). The arrangement of Ca\textsuperscript{2+} sensitive signaling molecules on scaffoldings close to the mouth of Ca\textsuperscript{2+} permeable ion channels accounts for the selective activation of NFAT by Ca\textsuperscript{2+} entry via L-type Ca\textsuperscript{2+} channels (Sattler et al. 1999; Weick et al. 2003). We speculate that localization of PMCA\textsubscript{2} to specific Ca\textsuperscript{2+} microdomains enables selective modulation of Ca\textsuperscript{2+} pumps to serve local needs.
**Conclusion**

The principal finding from this study is that the mechanism of \(Ca^{2+}\)-dependent modulation of the plasma membrane \(Ca^{2+}\) pump in hippocampal neurons depends on the source of \(Ca^{2+}\). Calpain is clearly an important regulator of PMCA function following large \([Ca^{2+}]_i\) increases. The differing effects of depolarization- versus NMDA-induced \([Ca^{2+}]_i\) increases on PMCA function suggest that \(Ca^{2+}\) clearance may be selectively modulated within \(Ca^{2+}\) channel microdomains in neurons.

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References


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Figure Legends

Figure 1: Experimental protocol for studying stimulus-induced changes in PMCA function in hippocampal neurons.

A: Representative recording from a hippocampal neuron displays the full protocol used in these experiments. Small, brief increases in [Ca\(^{2+}\)]\(_i\) (test responses) were produced by trains of action potentials elicited by electric field stimulation (↑). Cyclopiazonic acid (CPA; 5 µM) was applied at the time indicated and maintained throughout the experiment to block SERCA type Ca\(^{2+}\) pumps. Recovery of [Ca\(^{2+}\)]\(_i\) from test responses were fit with a mono-exponential function and the time constant (τ) calculated. The initial time constant (τ\(_0\)) was the average of two test responses immediately following treatment with CPA (b and c, t = 0 at c). The cell was then treated with a 10 min priming stimulus (starting at t = 2 min) which in this example was a change in [Mg\(^{2+}\)]\(_o\) in the superfusate from 0.9 to 0.1 mM. A test stimulus to assess PMCA function was delivered at 4 min intervals subsequent to return to pre-treatment resting levels of [Ca\(^{2+}\)]\(_i\). B: Test responses just before (red; a in A; τ = 1.5 s) and immediately after application of CPA (blue; b in A; τ = 3.0 s) are superimposed on an expanded time scale. Heavy lines are exponential fits to the [Ca\(^{2+}\)]\(_i\) recovery traces. Time 0 is taken as the time of the second test response after CPA treatment (c in A). C: Test responses recorded at times 0 (green; c in fig. 1A; τ = 2.9 s), 48 (red; d in fig. 1A; τ = 4.0 s), 76 (black; e in fig 1A; τ = 5.0 s) and 92 (blue; f in fig. 1A; τ = 6.1 s) min are superimposed on an expanded time scale. Heavy lines are exponential fits to the [Ca\(^{2+}\)]\(_i\) recovery traces.
Figure 2: PMCA-mediated $[Ca^{2+}]_i$ recovery kinetics are stable in untreated hippocampal neurons.

PMCA function was measured using the protocol presented in Fig. 1. A: In the absence of a priming stimulus the $[Ca^{2+}]_i$ remained at basal levels. B: Test responses recorded at times 0 (blue; $\tau = 5.0$ s), 52 (red; $\tau = 5.1$ s), and 76 (black; $\tau = 5.0$ s) min are superimposed on an expanded time scale. C: The time constant for $[Ca^{2+}]_i$ recovery ($\tau$) was normalized to the initial time constant ($\tau_o$) prior to application of the priming stimulus (square) and plotted versus time. Plots are from representative cells recorded in the absence (open circles) and presence (triangles) of 10 $\mu$M calpeptin.
Figure 3: 0.1 mM [Mg$^{2+}$]$_o$-induced [Ca$^{2+}$]$_i$ spiking evoked calpain-mediated loss of PMCA activity.

PMCA function was measured using the protocol presented in Fig. 1. A: A priming stimulus in which the [Mg$^{2+}$]$_o$ in the superfusate was changed from 0.9 to 0.1 mM evoked repetitive [Ca$^{2+}$]$_i$ spikes. Trace is from time 2-12 min from the recording shown in Fig. 1A. B: The time constant for [Ca$^{2+}$]$_i$ recovery ($\tau$) was normalized to the initial time constant ($\tau/\tau_o$) prior to application of the 0.1 mM [Mg$^{2+}$]$_o$ priming stimulus (square) and plotted versus time. Plots are from representative cells recorded in the absence (open circles) and presence (triangles) of 10 µM calpeptin. C: Calpeptin did not affect 0.1 mM [Mg$^{2+}$]$_o$-induced [Ca$^{2+}$]$_i$ spiking. D: Test responses recorded at times 0 (blue; $\tau = 11.7$ s), 32 (red; $\tau = 9.2$ s), and 68 (black; $\tau = 5.9$ s) min from a cell treated with calpeptin and primed with 0.1 mM [Mg$^{2+}$]$_o$ are superimposed on an expanded time scale. Heavy lines are exponential fits to the data.
**Figure 4: NMDA-evoked calpain-mediated loss of PMCA activity.**

PMCA function was measured using the protocol presented in Fig. 1. A: An NMDA (100 µM) priming stimulus evoked a large increase in \([Ca^{2+}]_i\). B: Test responses recorded at times 0 (red; \(\tau = 2.8\)), 48 (black; \(\tau = 4.2\)), and 76 (blue; \(\tau = 5.5\)) min following priming with 0.1 mM \([Mg^{2+}]_o\) are superimposed on an expanded time scale. Heavy lines are exponential fits to the data. C: Calpeptin did not affect the NMDA induced \([Ca^{2+}]_i\) increase. D: Test responses recorded at times 0 (black; \(\tau = 5.3\) s), 36 (red; \(\tau = 3.3\) s), and 68 (blue; \(\tau = 5.2\) s) minutes from a cell treated with calpeptin and primed with 100 µM NMDA are superimposed on an expanded time scale. E: The time constant for \([Ca^{2+}]_i\) recovery (\(\tau\)) was normalized to the initial time constant (\(\tau/\tau_o\)) prior to application of the NMDA priming stimulus (square) and plotted versus time. Plots are from representative cells recorded in the absence (open circles) and presence (triangles) of 10 µM calpeptin.
Figure 5: Depolarization-induced calpain-mediated modulation of PMCA activity.

PMCA function was measured using the protocol presented in Fig. 1. A: A depolarizing priming stimulus of 30 mM [K⁺]₀ evoked a large increase in [Ca²⁺]. B: Test responses recorded at times 0 (red; τ = 3.4 s), 36 (black; τ = 2.7 s), and 56 (blue; τ = 3.0 s) minutes following priming with 30 mM [K⁺]₀ are superimposed on an expanded time scale. Heavy lines are exponential fits to the data. C: Calpeptin did not affect the 30 mM [K⁺]₀-induced [Ca²⁺]ᵢ increase. D: Test responses recorded at times 0 (red; τ = 5.6 s) and 60 (black; τ = 3.9 s) from a cell treated with calpeptin and primed with 30 mM [K⁺]₀ are superimposed on an expanded time scale. Heavy lines are exponential fits to the data. E: The time constant for [Ca²⁺]ᵢ recovery (τ) was normalized to the initial time constant (τ/τ₀) prior to the depolarizing priming stimulus (square) and plotted versus time. Plots are from representative cells (shown in A-B and C-D) recorded in the absence (open circles) and presence (triangles) of 10 µM calpeptin.
Figure 6. Ca\(^{2+}\) source specific modulation of PMCA function.

(A-C) Bar graphs summarize pooled data from time-dependent changes in \(\tau/\tau_0\) evoked by the following priming stimuli: none (control), depolarization (30 mM [K\(^+\)]\(_o\)), excitatory synaptic activity (0.1 mM [Mg\(^{2+}\)]\(_o\)) and 100 µM NMDA. # and * symbols only indicate significant comparison (univariate ANOVA with Tukey’s post-hoc test) to the matching control condition. # indicates untreated condition, and * indicates 10 µM calpeptin condition (* or # p<0.05; ** or ## p<0.01). All significant comparisons noted below.

A: Mean \(\tau/\tau_0\). Only in the untreated condition did priming stimuli differ from each other, 
# ((F3,15)=11.22, p<0.001). The means of the control and 30 mM [K\(^+\)]\(_o\) are both smaller than 0.1 mM [Mg\(^{2+}\)]\(_o\) (p<0.05, p<0.01) and NMDA (p<0.01, p<0.001). The difference between untreated and calpeptin means (t-test with equal variance not assumed) was significant for 0.1 mM [Mg\(^{2+}\)]\(_o\) (t5.6 = 4.07; p<0.01) and NMDA (t8.7 = 4.44, p<0.001) primes. B: The slope of the linear regression. Slopes of the time-dependent changes in \(\tau/\tau_0\) following priming stimuli differed in the untreated condition ((F3,13)=5.35, p<0.05)), with the control slope smaller than the other three conditions (p<0.05); and in the calpeptin condition ((F3,12)=15.03, p<0.001), the NMDA slope is different from the other three (p<0.01), and the 0.1 mM [Mg\(^{2+}\)]\(_o\) slope was different than control (p<0.01). Comparison of untreated and calpeptin shows significant differences between slopes of 30 mM [K\(^+\)]\(_o\) (t4=4.91, p<0.01), 0.1 mM [Mg\(^{2+}\)]\(_o\) (t3.7=5.62, p<0.01) and NMDA (t5.5=2.4, p<0.05). C: Y-intercept of the linear regression. Y-intercept of the regression fit to time-dependent changes in \(\tau/\tau_0\) following priming stimuli differed in the untreated condition (F(3,13)=6.7, p<0.01), the line fit to kinetics data following 30 mM [K\(^+\)]\(_o\) had a
smaller intercept relative to the other conditions (p<0.01); and in the calpeptin condition ((F3,12)=6.16, p<0.01)) the NMDA intercept was smaller than the other three (p<0.01). Error bars indicate ± SEM.