Cellular/Molecular

# Activation of Protein Kinase C in Sensory Neurons Accelerates $Ca^{2+}$ Uptake into the Endoplasmic Reticulum

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**The rate of $Ca^{2+}$ clearance from the neuronal cytoplasm affects the amplitude, duration, and localization of $Ca^{2+}$ signals and influences a variety of $Ca^{2+}$-dependent functions. We reported previously that activation of protein kinase C (PKC) accelerates $Ca^{2+}$ efflux in rat sensory neurons mediated by the plasma membrane $Ca^{2+}$-ATPase isoform 4 (PMCA4). Here we show that sarco-endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA)-mediated $Ca^{2+}$ uptake into intracellular stores is also accelerated by PKC activation. The rate of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) clearance was studied after small (<350 nM) action potential-induced $Ca^{2+}$ loads in rat dorsal root ganglion neurons. Under these conditions, mitochondrial $Ca^{2+}$ uptake and $Na^+/Ca^{2+}$ exchange do not significantly influence $[Ca^{2+}]_i$ recovery. Phorbol dibutyrate (PDBu) increased the rate of $[Ca^{2+}]_i$ clearance by 71% in a manner sensitive to the selective PKC inhibitors GF109203x (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide) and calphostin. PKC-dependent acceleration was still observed (~39%) when the PKC-sensitive PMCA isoform was knocked down by expression of an antisense PMCA4 cDNA (AS4). Direct measurement of $Ca^{2+}$ in the endoplasmic reticulum (ER) lumen revealed that PKC increased the rate of store refilling more than twofold after depletion by treatment with cyclopiazonic acid. ER refilling was less complete in PDBu-treated cells, although, in AS4-expressing cells, PDBu accelerated the rate without reducing the ER capacity, suggesting that PMCA and SERCA compete for $Ca^{2+}$. Thus, activation of PKC accelerates the clearance of $Ca^{2+}$ from the cytoplasm by the concerted stimulation of $Ca^{2+}$ sequestration and $Ca^{2+}$ efflux.**

*Key words:* PKC; SERCA; PMCA; ER; $Ca^{2+}$ uptake; Mag-indo-1; sensory neuron

## Introduction

Phosphorylation of voltage- and ligand-gated $Ca^{2+}$ channels is a critical means to regulate $Ca^{2+}$ signaling and neuronal plasticity (Catterall, 2000; Salter and Kalia, 2004). In contrast, little is known about the modulation of the $Ca^{2+}$ clearance mechanisms counterbalancing $Ca^{2+}$ influx pathways. The high-affinity $Ca^{2+}$ pumps in the endoplasmic reticulum (ER) and plasma membrane are especially important for recovery of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) after brief electrical stimuli (Benham et al., 1992; Werth et al., 1996). Recently, we found that $Ca^{2+}$ extrusion was stimulated by activation of protein kinase C (PKC) in sensory neurons, and that this effect was mediated by the plasma membrane $Ca^{2+}$ ATPase (PMCA) isoform 4b (Usachev et al., 2002). In this report, we examine the possibility that $Ca^{2+}$ sequestration might also be stimulated by PKC.

$Ca^{2+}$ uptake mediated by the sarco-endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) significantly contributes to $Ca^{2+}$ clearance in neurons. Accordingly, the rate of $Ca^{2+}$ sequestration into the ER modulates the amplitude and duration of cytosolic $Ca^{2+}$ signals (Neering and McBurney, 1984; Friel and Tsien, 1992; Shmigol et al., 1994; Fierro et al., 1998; Suzuki et al., 2002). SERCA also functions to replenish ER stores with releasable $Ca^{2+}$ (Garaschuk et al., 1997; Usachev and Thayer, 1999), which determines the ability of the stores to amplify cytosolic $Ca^{2+}$ signals. $Ca^{2+}$ release via ryanodine or inositol 1,4,5-trisphosphate ($IP_3$) receptors regulates excitability, neuronal differentiation, and some forms of synaptic plasticity (for review, see Berridge, 1998; Verkhratsky, 2005). Furthermore, $Ca^{2+}$ sequestered within the ER is essential for maintaining protein synthesis (Brostrom and Brostrom, 2003), whereas chronic $Ca^{2+}$ overload of the stores is implicated in some neurological disorders (Paschen, 2003; Verkhratsky, 2005). Thus, SERCA-mediated $Ca^{2+}$ sequestration is a critical component of neuronal signaling.

In neurons, modulation of SERCA-type $Ca^{2+}$ pumps by kinases has not been described. However, in muscle, protein kinase A (PKA) and calmodulin-dependent kinase (CaMK) phosphorylate phospholamban, a SERCA accessory protein, to accelerate SERCA-mediated $Ca^{2+}$ uptake into the sarcoplasmic reticulum (Tada and Toyofuku, 1998; MacLennan and Kranias, 2003). Phospholamban is not expressed in neurons (Plessers et al., 1991), although analogous proteins may be present (Dou and Joseph, 1996). Modulation of $Ca^{2+}$ sequestration in the ER by phosphorylation has also been reported in blood platelets and in *Xenopus* oocytes (Lacabaratz-Porret et al., 1998; Roderick et al.,

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2000). Thus, although little is known about the modulation of SERCAs in neurons, in other cell types, SERCA-mediated $Ca^{2+}$ uptake is modulated by protein kinases.

Here, we found that activation of PKC stimulated $Ca^{2+}$ clearance from the cytoplasm of sensory neurons. PKC-dependent facilitation of $Ca^{2+}$ extrusion (Usachev et al., 2002) could not alone account for this marked acceleration of $[Ca^{2+}]_i$ recovery. In addition, SERCA-mediated $Ca^{2+}$ uptake significantly contributed to the PKC effect. Direct monitoring of $Ca^{2+}$ concentration in the ER revealed a more than twofold increase in the rate of $Ca^{2+}$ sequestration into the lumen of the ER. Thus, the activation of PKC results in a coordinated acceleration of multiple $Ca^{2+}$ clearance mechanisms in sensory neurons.

## Materials and Methods

*Materials.* Indo-1 AM, Mag-indo-1 AM, and Pluronic F-127 were obtained from Invitrogen (Carlsbad, CA). GF109203x (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide), Ro31-8220 (3-[1-(3-(amidinothio)propyl)-1*H*-indol-3-yl]-3-(1-methyl-indol-3-yl)maleimide), calphostin, and phorbol dibutyrate (PDBu) were obtained from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO).

*Cell culture.* Rat DRG neurons were grown in culture as described previously (Werth et al., 1996). In brief, 1- to 3-d-old Sprague Dawley rats were killed under a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee. DRGs were dissected from the thoracic and lumbar segments and incubated at 37°C in collagenase–dispase (0.8 and 6.4 U/ml, respectively) for 45 min. Ganglia were dissociated by trituration through a flame-constricted pipette and then plated onto laminin-coated (50 μg/ml) glass coverslips (25 mm diameter). Cells were grown in Ham's F-12 media supplemented with 5% heat-inactivated horse serum and 5% fetal bovine serum, 50 ng/ml NGF, 4.4 mM glucose, 2 mM L-glutamine, modified Eagle's medium vitamins, and penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively). Cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$. Neurons with cell body diameters of 18–27 μm were used within 2–3 d of plating.

*$[Ca^{2+}]_i$ measurements.* Instrumentation for $[Ca^{2+}]_i$ recording from single DRG neurons using indo-1- or Mag-indo-1-based microfluorimetry was similar to that described previously (Werth et al., 1996). Cells were placed in a flow-through chamber that was mounted on the stage of an inverted epifluorescence microscope equipped with a 70× objective (numerical aperture, 1.15; Leitz, Wetzlar, Germany). To introduce the indicators into cells, the culture was incubated with the AM form of indo-1 at a concentration of 10 μM at room temperature (22°C) for 30 min or the AM form of Mag-indo-1 at a concentration of 7.5 μM in 0.02% Pluronic F-127 at 37°C for 30 min. The cells were washed for 30 min in dye-free HEPES-buffered Hank's salt solution (HHSS) at 37°C before initiating recording. HHSS had the following composition (in mM): 10 HEPES, 140 NaCl, 5 KCl, 1.3 $CaCl_2$, 0.4 $MgSO_4$, 0.5 $MgCl_2$, 0.4 $KH_2PO_4$, 0.6 $Na_2HPO_4$, 3 $NaHCO_3$, and 10 glucose, pH 7.35 with NaOH (310 mOsm/kg with sucrose). $Ca^{2+}$-free solution was obtained by substituting 0.1 mM EGTA for $Ca^{2+}$. Indo-1 and Mag-indo-1 were 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. Changes in the fluorescence of indo-1 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 fluorescent intensity ratio (Grynkiewicz et al., 1985). The dissociation constant used 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 $Ca^{2+}$. $R_{min}$, $R_{max}$, and $\beta$ were determined in intact cells by applying 10 μM ionomycin in $Ca^{2+}$-free buffer (1 mM EGTA) and saturating $Ca^{2+}$ (5 mM $Ca^{2+}$). Values for $R_{min}$, $R_{max}$, and $\beta$ were 1.16, 10.4, and 4.3, respectively. Potential cytoplasmic contamination with Mag-indo-1 prevented us from calibrating the indicator; thus, we reported $[Ca^{2+}]_{ER}$ as the ratio ($R$) of fluorescence intensity of the $Ca^{2+}$-bound (405 nm) relative to the $Ca^{2+}$-free form (490 nm) of the dye. To evoke action potentials in intact neurons, extracellular field stimulation was used (Piser et al., 1994). Exponential functions were fitted to the data using a nonlinear, least-squares curve fitting algorithm (Origin 4.1 software; OriginLab, Northampton, MA). Data are presented as mean ± SEM.

*Antisense experiments.* Gene transfer into DRG neurons was performed using a biolistic particle delivery system as described previously (Usachev et al., 2000). A mammalian expression plasmid (pCI-neo) harboring cDNAs encoding nucleotides 71–443 of PMCA4 in the antisense orientation (AS4) was generated as described previously (Garcia et al., 2001). The pCI-neo-based construct was mixed in a 4:1 ratio with a plasmid encoding enhanced green fluorescent protein (pEGFP-C1; Clontech, Mountain View, CA) and precipitated on 1.6 μm gold particles. After 48 h, transfected cells were identified by green fluorescence [excitation, 480(10) nm; emission, 540(25) nm]. Effective knockdown of PMCA4 was confirmed by immunohistochemistry with PMCA4-specific antibody JA9, as described previously (Usachev et al., 2002).

## Results

In sensory neurons, a small increase in $[Ca^{2+}]_i$ recovers to basal levels by the concerted action of $Ca^{2+}$ efflux and sequestration processes (Benham et al., 1992; Werth et al., 1996; Usachev and Thayer, 1999). Rat DRG neurons in culture were challenged with small $Ca^{2+}$ loads by firing brief trains of action potentials (3–5 s, 6–10 Hz) using electric field stimulation (Piser et al., 1994), and changes in $[Ca^{2+}]_i$ were monitored with indo-1-based photometry (Werth and Thayer, 1994). This stimulus is within the physiological range for DRG neurons (Matthews, 1931; Lawson, 2002), evokes a rapid increase in $[Ca^{2+}]_i$ that minimizes the effect of $Ca^{2+}$ buffering on amplitude, and results in peak $[Ca^{2+}]_i$ between 200 and 350 nM, which is the range in which ATPases dominate $Ca^{2+}$ clearance. The rate constant for recovery ($k$), a value reciprocal to the time constant, was calculated for each field stimulation-evoked $[Ca^{2+}]_i$ signal by fitting the $[Ca^{2+}]_i$ recovery phase to a monoexponential decay function. As shown in Figure 1, a brief train of action potentials evoked increases in $[Ca^{2+}]_i$ that were reproducible in amplitude and recovery rate. We have shown previously that mitochondrial $Ca^{2+}$ uptake and $Na^+/Ca^{2+}$ exchange do not significantly influence the rate of recovery from these small (<350 nM) increases in $[Ca^{2+}]_I$ (Werth and Thayer, 1994; Usachev et al., 2002). However, when PMCA- and SERCA-type $Ca^{2+}$ pumps were poisoned with vanadate, $[Ca^{2+}]_i$ recovery kinetics slowed by more than ninefold (Usachev et al., 2002).

### Activation of PKC accelerates $Ca^{2+}$ clearance from DRG neurons

Treatment of DRG neurons with 500 nM PDBu, an activator of PKC, significantly accelerated the clearance of $Ca^{2+}$ from the cytoplasm (Fig. 1*A*,*B*). PDBu inhibited whole-cell $Ca^{2+}$ currents by 32 ± 3% ($n = 4$), consistent with previous reports (Rane and Dunlap, 1986; Gross and MacDonald, 1989; Boland et al., 1991; Diversepierluissi and Dunlap, 1993) (but see Hall et al., 1995). When peak $[Ca^{2+}]_i$ was maintained constant by increasing the stimulus intensity in the presence of PDBu, the rate of $Ca^{2+}$ recovery ($k$) increased from 4.3 ± 0.4 to 7.3 ± 0.7 $min^{-1}$ over ~20 min ($n = 11$; $p < 0.001$ for $t \geq 15$ min; repeated measures ANOVA, Bonferroni's *post hoc* test). This corresponds to an increase in rate constant to 171 ± 10% of control (Fig. 1*C*). When stimulus strength was held constant throughout the recording, the amplitude of the $[Ca^{2+}]_i$ response decreased from 272 ± 39 nM ($t = 0$) to 221 ± 35 nM ($t = 24$ min; $n = 7$), and the rate constant for $Ca^{2+}$ clearance increased to 175 ± 14% ($n = 7$) during treatment with PDBu. The stimulation of $Ca^{2+}$ clearance

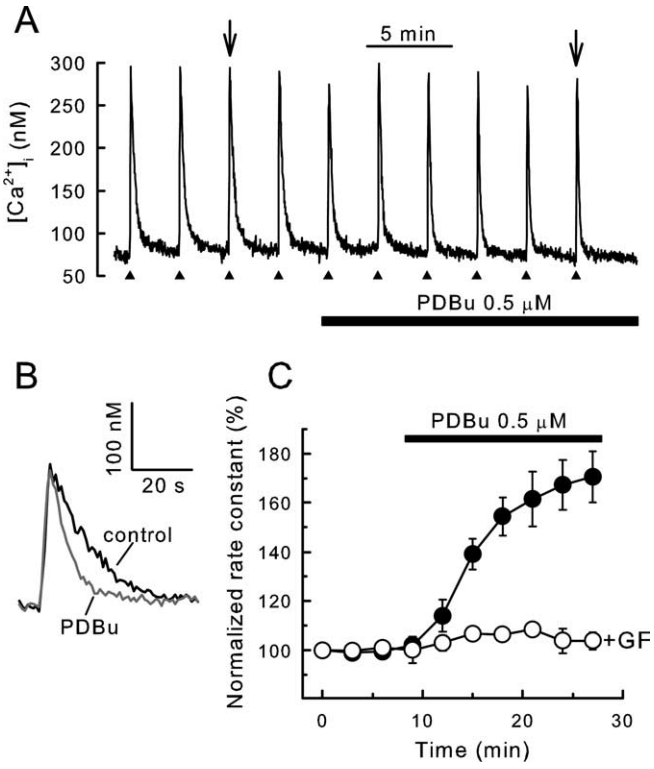


**Figure 1.** Activation of PKC accelerates recovery from action potential-induced increases in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was recorded from single rat DRG neurons using indo-1-based photometry as described in Materials and Methods. ***A***, Brief trains of action potentials (3–5 s, 6–10 Hz) were delivered every 3 min as indicated by the filled triangles, and 0.5 $\mu$M PDBu was applied to the bath during the time indicated by the horizontal bar. To maintain comparable $Ca^{2+}$ loads throughout the experiment, stimulation frequency in the presence of PDBu was increased by 20%. ***B***, Representative $[Ca^{2+}]_i$ responses in the absence and presence of PDBu, indicated by arrows in ***A***, were superimposed on an expanded timescale. ***C***, Rate constants describing the recovery kinetics for individual responses from experiments such as the one shown in ***A*** were normalized to the first response and plotted versus time. PDBu was applied after the fourth stimulus. Experiments were performed in the absence (filled circles; $n = 11$) and presence (open circles; $n = 5$) of 5 $\mu$M GF109203x (GF) applied at time 0. Data points are means ± SEM.

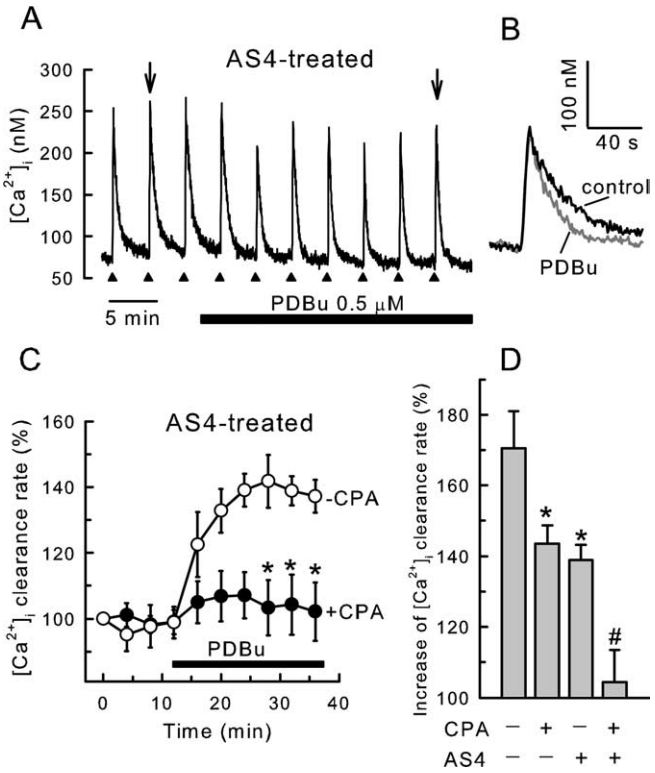


**Figure 2.** PDBu stimulates SERCA-mediated recovery from action potential-induced increases in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was recorded from single rat DRG neurons using indo-1-based photometry. ***A–D***, DRG neurons were cotransfected with plasmids containing an EGFP reporter construct and an antisense PMCA4 cDNA (AS4). Recordings are from EGFP-positive cells. ***A***, Brief trains of action potentials (3–5 s, 6–10 Hz) were delivered every 4 min to an AS4-expressing cell as indicated by the filled triangles. PDBu at 0.5 $\mu$M was applied to the bath during the time indicated by the horizontal bar. ***B***, Representative $[Ca^{2+}]_i$ responses in the absence and presence of PDBu, indicated by arrows in ***A***, were superimposed on an expanded timescale. ***C***, Rate constants describing the recovery kinetics for individual responses from experiments such as the one shown in ***A*** were normalized to the first response and plotted versus time. PDBu was applied after the fourth stimulus. Experiments were performed in the absence (open circles; $n = 5$) and presence (filled circles; $n = 11$) of 5 $\mu$M CPA applied 30 min before beginning the recording. Data points are means ± SEM. $^*p < 0.05$ relative to the same time point when CPA was not added, one-way ANOVA with Bonferroni's *post hoc* test. Results in the presence of CPA are replotted from Usachev et al. (2002). ***D***, Bar graph summarizes results of various combinations of SERCA and PMCA4 block on the PDBu effect. $^*p < 0.05$, relative to untreated control (no AS4 and no CPA); $^{\#}p < 0.05$ relative to cells expressing AS4 (no CPA); Student's *t* test.

was completely blocked by the PKC antagonists GF109203x (5 $\mu$M; $n = 5$) (Fig. 1*C*) and calphostin (300 nM; $n = 4$; data not shown). Another phorbol ester, phorbol-12-myristate-13-acetate (PMA) (1 $\mu$M; 18 min) produced a similar increase in the $[Ca^{2+}]_i$ recovery rate (158 ± 15%; $n = 7$). In contrast, the inactive analog 4-$\alpha$-PMA (1 $\mu$M) was without effect (105 ± 3%; $n = 4$).

We have shown previously that activation of PKC accelerated $Ca^{2+}$ efflux by ~45% via PMCA4 in DRG neurons with SERCAs blocked by treatment with cyclopiazonic acid (CPA) (Usachev et al., 2002). For small $Ca^{2+}$ loads, CPA reduced the rate constant from 4.9 ± 0.5 to 1.9 ± 0.3 $min^{-1}$ ($n = 8$), suggesting that, under these conditions, SERCA accounted for ~60% of $Ca^{2+}$ clearance in DRG neurons. The remaining $Ca^{2+}$ was primarily removed from the cytoplasm by PMCAs (Werth and Thayer, 1994; Usachev et al., 2002). If PMCA was the only $Ca^{2+}$ transporter targeted by PKC, then the effect of PDBu should be diluted when SERCA was not blocked by CPA. In contrast, we found that, in the absence of CPA, PKC-dependent acceleration of $[Ca^{2+}]_i$ recovery was more pronounced (171 ± 10% of control; $n = 11$) (Fig. 1*C*), suggesting that PKC might also stimulate another $Ca^{2+}$ clearance process.

### Activation of PKC stimulates PMCA4 and SERCA-dependent $Ca^{2+}$ clearance

To determine the contribution of SERCA-mediated $Ca^{2+}$ uptake into the ER to PKC-stimulated acceleration of $Ca^{2+}$ clearance from the cytoplasm, we examined $[Ca^{2+}]_i$ recovery kinetics in cells in which PMCA4, the PKC-sensitive plasma membrane $Ca^{2+}$ pump isoform, was knocked down by expression of a PMCA4 antisense cDNA (AS4). We have shown previously that, in DRG neurons cotransfected with EGFP reporter and AS4 expression plasmids, PMCA4 immunoreactivity was undetectable (Usachev et al., 2002). In Figure 2, *A* and *B*, we show that activation of PKC increased the rate of $[Ca^{2+}]_i$ recovery to 139 ± 4% of control ($n = 5$) in cells with reduced PMCA4 protein. Basal $[Ca^{2+}]_i$ in control (63 ± 7 nM; $n = 11$) and AS4-expressing (66 ± 5 nM; $n = 5$) cells were similar. In AS4-expressing cells, $[Ca^{2+}]_i$ recovery kinetics were not affected by the mitochondrial uncoupling agent FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) (1 $\mu$M) in either the absence (5.2 ± 0.3 and 5.2 ± 0.2 $min^{-1}$, without or with FCCP, respectively; $n = 4$) or

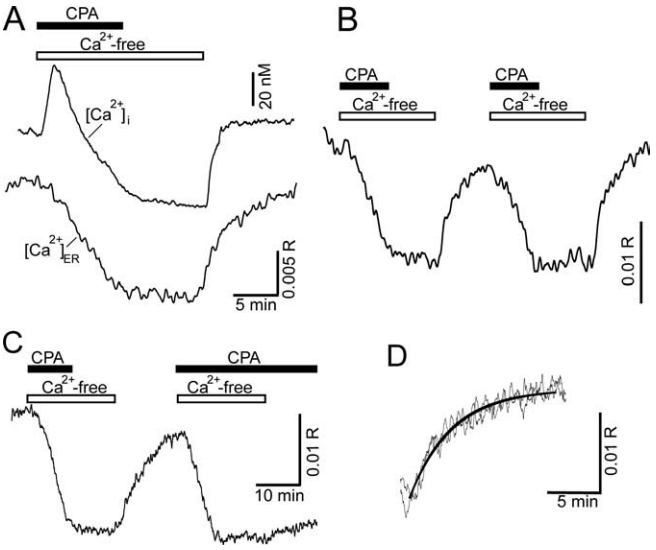


**Figure 3.** Direct recording of $[Ca^{2+}]_{ER}$ to measure SERCA function. $[Ca^{2+}]$ in the lumen of the ER was recorded using Mag-indo-1, and $[Ca^{2+}]_i$ was recorded using indo-1. ***A***, Representative $[Ca^{2+}]_i$ (top trace) and $[Ca^{2+}]_{ER}$ (bottom trace) recordings obtained from two different cells are plotted on the same timescale. Depleting the $Ca^{2+}$ stores by blocking SERCA with 5 $\mu$M CPA in $Ca^{2+}$-free buffer (indicated by horizontal bars) produced a leak of $Ca^{2+}$ from the ER, resulting in a decrease in $[Ca^{2+}]_{ER}$ and a corresponding increase in $[Ca^{2+}]_i$. Return of $Ca^{2+}$ to the media allowed the ER to refill with $Ca^{2+}$. ***B***, Representative $[Ca^{2+}]_{ER}$ recording shows that the refilling process could be evoked repeatedly. ***C***, If CPA was not removed before the second application of $Ca^{2+}$ to the bath, refilling was completely blocked. ***D***, The two recovery phases from ***B*** were superimposed. The $[Ca^{2+}]_{ER}$ recovery was well described by a single-exponential equation (heavy black line).

presence (7.6 ± 0.5 and 7.4 ± 0.7 $min^{-1}$, without or with FCCP, respectively; $n = 4$) of PDBu. In PMCA4-deficient cells, CPA essentially blocked stimulation by PDBu (104 ± 9%; $n = 11$) (Fig. 2*C*). In contrast, in cells with normal levels of PMCA4, CPA reduced the maximal response in PDBu from 171 ± 10% ($n = 11$) to 143 ± 5% ($n = 14$). Thus, activation of PKC stimulates PMCA4-mediated $Ca^{2+}$ efflux and SERCA-mediated $Ca^{2+}$ uptake (Fig. 2*D*).

### Activation of PKC increases the rate of $Ca^{2+}$ sequestration

We tested further the hypothesis that activation of PKC stimulates $Ca^{2+}$ uptake into the ER by measuring ER $Ca^{2+}$ levels ($[Ca^{2+}]_{ER}$) directly. DRG neurons were loaded with the low-affinity $Ca^{2+}$ indicator Mag-indo-1 ($K_d \approx 35$ $\mu$M) under conditions that preferentially loaded the ER, similar to the protocols using another low-affinity $Ca^{2+}$ dye Mag-fura-2 to monitor $[Ca^{2+}]_{ER}$ in neurons (Fujiwara et al., 2001; Solovyova et al., 2002; Solovyova and Verkhratsky, 2003). Because of the low affinity of this indicator, any dye localized to the cytoplasm did not appreciably contribute to $Ca^{2+}$-dependent changes in fluorescence. Thus, when the ER was depleted and refilled with $Ca^{2+}$, indo-1-based $[Ca^{2+}]_i$ and Mag-indo-1-based $[Ca^{2+}]_{ER}$ recordings displayed complimentary waveforms (Fig. 3*A*). Application of CPA (5 $\mu$M) in $Ca^{2+}$-free media produced a slow leak of $Ca^{2+}$ from the ER into the cytoplasm (Camello et al., 2002). $[Ca^{2+}]_i$ returned to a lower baseline as $Ca^{2+}$ was pumped out of the cell across the plasma membrane. After washing CPA from the cell, return of $Ca^{2+}$ to the media produced a small increase in $[Ca^{2+}]_i$ and a marked refilling of the ER lumen. The $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ER}$ traces in Figure 3*A* were each collected from a separate cell and aligned temporally. We also performed simultaneous $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ER}$ measurements using fura-2 and Mag-fluo-4. The combined recordings were in excellent agreement with Figure 3*A* and are displayed in supplemental Figure 1 (available at www.jneurosci.org as supplemental material). These data show that the two indicators were clearly detecting $Ca^{2+}$ in different pools. Changes in $[Ca^{2+}]_i$ correlated with the rate of $Ca^{2+}$ mobilization from intracellular stores ($d[Ca^{2+}]_{ER}/dt$) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This observation suggests that $[Ca^{2+}]_i$ is the net result of simultaneous $Ca^{2+}$ mobilization from ER stores and $Ca^{2+}$ extrusion across the plasma membrane. The rate constant of $[Ca^{2+}]_{ER}$ recovery was derived from an exponential equation fit to the time course of refilling and used as a quantitative index of the rate of $Ca^{2+}$ uptake into the ER. The mean rate constant ($k$) for refilling was 0.33 ± 0.05 $min^{-1}$ ($n = 15$) and is in good agreement with the rate of store refilling assessed by discharge with caffeine (Usachev and Thayer, 1999).

We used a paired protocol to assess the modulation of refilling (Fig. 3*B*). The ER could be repeatedly depleted, and the rate of refilling was remarkably reproducible for a given cell ($k2/k1 = 1.07 \pm 0.08$; $n = 15$) (Fig. 3*D*). The initial $[Ca^{2+}]_{ER}$ varied widely from cell to cell, although the amplitude of $[Ca^{2+}]_{ER}$ changes ($\Delta[Ca^{2+}]_{ER}$), defined as the difference between the stable asymptote reached after refilling and the $[Ca^{2+}]$ in depleted stores, reached consistent values under the controlled conditions of the store replenishment protocol. After the second refilling period, $\Delta[Ca^{2+}]_{ER}$ reached 118 ± 8% ($n = 15$) of the value after the first refilling. This $Ca^{2+}$ reuptake by the stores was mediated by SERCA-type $Ca^{2+}$ pumps because this process was blocked by 5 $\mu$M CPA (Fig. 3*C*) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). We next used this method to determine the effects of PKC activation on SERCA-mediated refilling of the ER with $Ca^{2+}$.

Application of PDBu (0.5 $\mu$M) before the second refilling in the paired protocol significantly accelerated the rate of $Ca^{2+}$ uptake (Fig. 4*A*,*B*). In the presence of PDBu, the rate of refilling increased to 275 ± 32% ($n = 10$) of the initial control rate. Interestingly, PDBu also reduced the level of the $Ca^{2+}$ store replenishment. In Figure 4*B*, refilling traces in the absence and presence of PDBu are shown superimposed. $[Ca^{2+}]_{ER}$ returned to only 77 ± 4% ($n = 10$) of control levels in the presence of PDBu ($p < 0.01$; $n = 10$) (Fig. 4*E*). These effects of PDBu resulted from the activation of PKC as indicated by a complete block of the acceleration in the presence of the PKC antagonist Ro31-8220 (Fig. 4*C*,*D*).

To determine whether PKC increased SERCA-mediated $Ca^{2+}$ transport into the ER or whether it reduced the leak of $Ca^{2+}$ out of the ER (Camello et al., 2002), we examined the effects of PDBu on the mobilization of $Ca^{2+}$ from loaded $Ca^{2+}$ stores. $[Ca^{2+}]_{ER}$ was recorded from DRG neurons after application of a high concentration (30 $\mu$M) of CPA to rapidly and completely block $Ca^{2+}$ uptake (Fig. 4*F*). The loss of ER $Ca^{2+}$ appeared to initially follow a linear process. PDBu had no effect on the slope of the linear regression fit to the initial phase of ER $Ca^{2+}$ mobilization. In control recordings, the slope of the second release phase was 83 ± 2% ($n = 4$) of the first. When PDBu was added before and during the second release phase, the slope was 80 ± 4% ($n = 3$) of the first phase, which was not significantly different from control. Thus, PDBu does not appear to influence the $Ca^{2+}$ leak from the ER, suggesting that PKC activation stimulates SERCA.

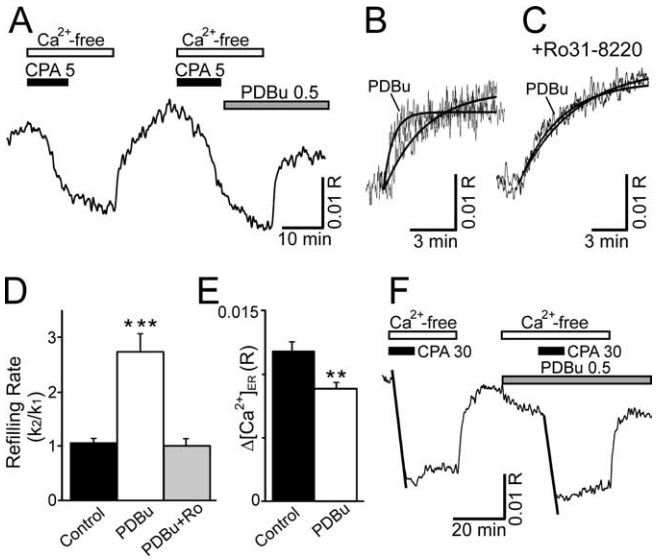


**Figure 4.** Activation of PKC accelerates $Ca^{2+}$ uptake into the ER. $[Ca^{2+}]$ in the lumen of the ER was recorded using Mag-indo-1-based photometry. ***A***, Representative recording shows the effects of PDBu on the rate of $[Ca^{2+}]_{ER}$ recovery using the refilling protocol described in Figure 3*B*. CPA at 5 $\mu$M, $Ca^{2+}$-free buffer, and 0.5 $\mu$M PDBu were applied to the bath by superfusion at the times indicated by the horizontal bars. ***B***, $[Ca^{2+}]_{ER}$ recordings from ***A*** were superimposed on an expanded timescale. Heavy lines show fitted single-exponential curves. ***C***, $[Ca^{2+}]_{ER}$ recordings from an experiment similar to that in ***A***, except that the PKC antagonist Ro31–8220 (2 $\mu$M) was applied 10 min before the application of PDBu. The initial control response and the response in the presence of Ro31-8220 plus PDBu were superimposed on an expanded timescale. Heavy lines show fitted single-exponential curves. ***D***, Bar graph summarizes the effects of PKC activation on refilling kinetics. ***$p < 0.001$ relative to untreated control, Student's *t* test. ***E***, Bar graph illustrates difference in completeness of refilling in the absence (control) and presence (0.5 $\mu$M) of PDBu. $\Delta[Ca^{2+}]_{ER}$ was defined as the difference between the stable asymptote reached after refilling and the $[Ca^{2+}]$ in depleted stores. **$p < 0.01$ relative to untreated control in same cell, Student's paired *t* test. ***F***, Representative recording shows the rapid depletion of $Ca^{2+}$ stores by the application of 30 $\mu$M CPA in $Ca^{2+}$-free media. The rate of $Ca^{2+}$ leaking from the store was quantified by measuring the slope of a linear regression fit to the first 5 min of the $[Ca^{2+}]_{ER}$ trace in CPA (fits are indicated by heavy lines).

### PKC-dependent competition between $Ca^{2+}$ extrusion and $Ca^{2+}$ sequestration

The traces in Figure 4*B* indicate that the activation of PKC increased the rate of $Ca^{2+}$ uptake into the ER but also reduced the total amount of $Ca^{2+}$ sequestered. We tested the hypothesis that PDBu was stimulating the PMCA, resulting in reduced $Ca^{2+}$ available for uptake into the ER. We first confirmed that less $Ca^{2+}$ was taken up into the ER when PKC was activated by using an alternative approach complimentary to the direct $[Ca^{2+}]_{ER}$ measurements in Figure 4. $Ca^{2+}$ release from the ER was evoked with CPA, and the increase in $[Ca^{2+}]_i$ was used as an index of the amount of stored $Ca^{2+}$. As shown in Figure 5*A*, 5 $\mu$M CPA elicited reproducible elevations in $[Ca^{2+}]_i$ when the cells were treated with the same refilling protocol as shown in Figure 3*B*. Treatment with PDBu during the second refilling phase (the same protocol as in Fig. 4*A*) decreased the amplitude of the CPA-evoked response (Fig. 5*A*,*B*). Thus, activation of PKC reduces the amount of $Ca^{2+}$ taken up into the ER. Resting $[Ca^{2+}]_i$ in naive cells was 53 ± 6 nM ($n = 16$) and declined significantly to 40 ± 5 nM during treatment with PDBu ($p < 0.001$, paired Student's *t* test). PMCA isoform 4 is also stimulated by PKC (Usachev et al., 2002). To determine whether PMCA4-mediated $Ca^{2+}$ efflux contributed to the reduced refilling of ER $Ca^{2+}$ stores in the presence of PDBu, PMCA4 was knocked down by expression of a PMCA4 antisense cDNA (AS4). In AS4-treated cells, the CPA-evoked $[Ca^{2+}]_i$ increase was comparable with nontransfected cells and cells transfected with EGFP expression vector alone (Fig. 5*C*). However, when PMCA4 expression was downregulated, PDBu no longer reduced the amplitude of the CPA-evoked response (Fig. 5*C*). Resting $[Ca^{2+}]_i$ in AS4 cells was 57 ± 9 nM and did not change significantly (50 ± 6 nM; $p = 0.25$, paired Stu-

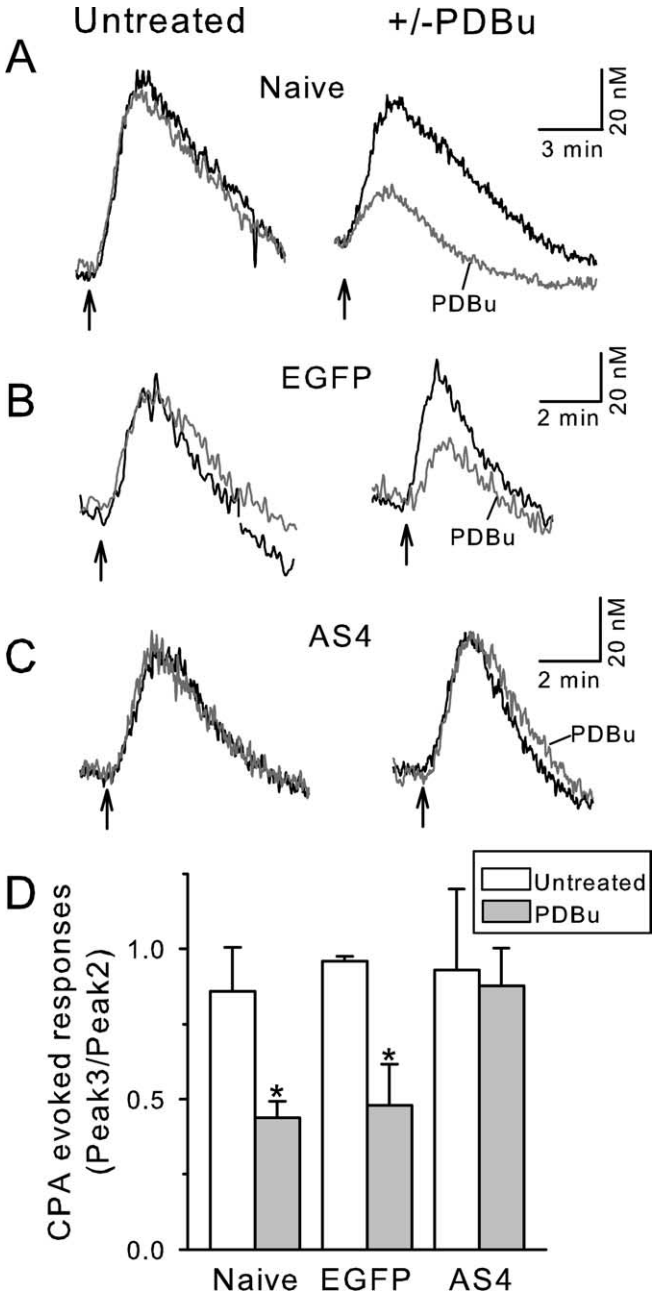


**Figure 5.** PKC activation reduces ER refilling by stimulating PMCA4. ***A–C***, $[Ca^{2+}]_i$ was recorded using indo-1-based photometry. CPA (5 $\mu$M) was applied in $Ca^{2+}$-free media at the times indicated by the arrows. Because the refilling status of the $Ca^{2+}$ stores varied at the start of the recording, the $[Ca^{2+}]_i$ transients shown in ***A–C*** are the second and third CPA-evoked responses and correspond to the refilling status of the store after the first and second refilling under controlled conditions. The duration of the treatments are identical to those in Figures 3 (left column) and 4 (right column). Representative traces from nontransfected (Naive) cells (***A***), cells transfected with EGFP (***B***), and cells transfected with EGFP plus AS4 (***C***) are shown. The second and third CPA-evoked responses were superimposed for untreated (left column) and PDBu-treated (right column) cells. ***D***, Bar graph summarizes the changes in the amplitude of the CPA-evoked response in the presence and absence of PDBu. *$p < 0.05$, PDBu compared with untreated, Student's *t* test.

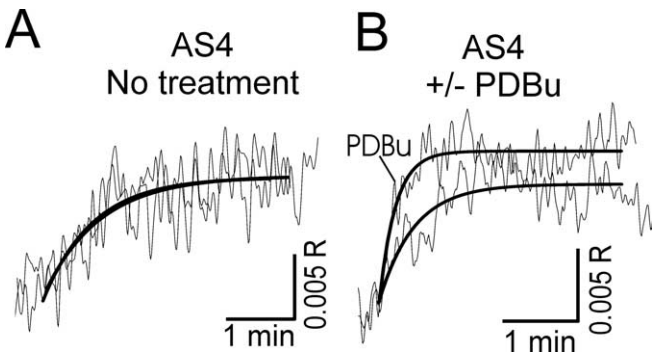


**Figure 6.** Activation of PKC accelerates the rate of ER refilling with $Ca^{2+}$ without reducing capacity in cells lacking the PKC-sensitive PMCA isoform. $[Ca^{2+}]_{ER}$ was recorded using Mag-indo-1-based photometry. DRG neurons were cotransfected with plasmids containing an EGFP reporter construct and an antisense PMCA4 cDNA. Recordings are from EGFP-positive cells. ***A***, Representative traces show $[Ca^{2+}]_{ER}$ in AS4-treated DRG neuron during repeated application of the refilling protocol described in Figure 3. The rate and degree of refilling were reproducible. ***B***, $[Ca^{2+}]_{ER}$ traces recorded from AS4-treated DRG neurons before and after PDBu application were superimposed. PDBu accelerated the rate of refilling and increased the steady-state $[Ca^{2+}]_{ER}$.

dent's *t* test) during treatment with PDBu ($n = 8$). Thus, PMCA4 appears to compete with the SERCA, depriving the ER of $Ca^{2+}$ when stimulated by PKC (Fig. 5*D*).

If both PMCA4- and SERCA-mediated $Ca^{2+}$ clearance were accelerated by activation of PKC, then in AS4-treated cells PDBu should accelerate the rate of ER $Ca^{2+}$ refilling without reducing the capacity of the ER. In AS4-treated cells the rate and completeness of refilling were reproducible (Fig. 6*A*). Furthermore, as our hypothesis predicted, activation of PKC in these cells accelerated the rate of $Ca^{2+}$ uptake into the ER ($k2/k1 = 1.9 \pm 0.3$; $n = 5$), without diminishing the steady level of refilling (Fig. 6*B*). In AS4-treated cells, the steady-state $[Ca^{2+}]_{ER}$ in the presence of PDBu was $126 \pm 11\%$ ($n = 5$) of control. Thus, both SERCA-mediated $Ca^{2+}$ sequestration and PMCA-mediated $Ca^{2+}$ efflux were accelerated by PKC, and these processes compete for $Ca^{2+}$.

## Discussion

We have shown that activation of PKC markedly accelerates the clearance of $Ca^{2+}$ from the cytoplasm of rat DRG neurons after physiological $Ca^{2+}$ loads. Our previous study identified $Ca^{2+}$ extrusion as one of the PKC targets (Usachev et al., 2002). Here we demonstrate PKC-dependent acceleration of $Ca^{2+}$ accumulation into the ER. This novel mechanism for regulating the $[Ca^{2+}]_i$ in neurons identifies ER $Ca^{2+}$ uptake as a point of crosstalk between $Ca^{2+}$ signaling and other second-messenger pathways. Furthermore, because PKC also modulates other elements of the $Ca^{2+}$ signaling system, these results indicate that $Ca^{2+}$ regulatory processes are controlled in a coordinated manner.

We used two complimentary approaches to study the acceleration of $[Ca^{2+}]_i$ clearance after activation of PKC. We first showed that the rate of $[Ca^{2+}]_i$ recovery from action potential-induced increases in $[Ca^{2+}]_i$ was increased by PDBu (Fig. 1) in a manner reversed by the PKC antagonists GF109203x and calphostin. The brief train of action potentials (3–5 s, 6–10 Hz) used as a stimulus is well within the physiological range of firing for sensory neurons (Matthews, 1931; Lawson, 2002), suggesting that PKC-dependent acceleration of $Ca^{2+}$ uptake is relevant to normal signal processing in these cells. The PKC-dependent acceleration of $Ca^{2+}$ clearance could be entirely accounted for by two processes: stimulation of PMCA4, a PKC-sensitive plasma membrane $Ca^{2+}$ pump isoform that was inhibited by antisense knockdown, and stimulation of a SERCA-mediated component that was inhibited by CPA (Fig. 2). Indeed, PDBu failed to accelerate $Ca^{2+}$ clearance in antisense 4-expressing cells treated with CPA. Note that recovery from the small $Ca^{2+}$ loads introduced by the stimulus used here are not significantly influenced by low-affinity $Ca^{2+}$ clearance mechanisms such as mitochondria and $Na^+/Ca^{2+}$ exchange (Werth and Thayer, 1994; Usachev et al., 2002). Recovery kinetics were not influenced by response amplitude within the range of peak $[Ca^{2+}]_i$ used in this study (Usachev et al., 2002).

The acceleration of $[Ca^{2+}]_i$ recovery kinetics via a CPA-sensitive process predicted that the rate of $Ca^{2+}$ uptake into the ER would also be increased. We tested this hypothesis by directly measuring $[Ca^{2+}]_{ER}$ with the low-affinity dye Mag-indo-1. The low affinity of this dye for $Ca^{2+}$ ($K_d \approx 35\ \mu M$) renders it virtually insensitive to the small changes in $[Ca^{2+}]_i$ observed in the protocols used here. Thus, even if some dye contaminated the cytoplasm, it could not account for the described effects of PDBu. Furthermore, our principal observation that PDBu increased the rate of rise of $[Ca^{2+}]_{ER}$ (Fig. 4) was opposite to the increased rate of recovery of $[Ca^{2+}]_i$ (Figs. 1, 2). The refilling protocol was initiated by the return of extracellular $Ca^{2+}$ to the bath. Thus, $Ca^{2+}$ influx across the plasma membrane could potentially influence the refilling rate. However, refilling kinetics were slower than the rise in $[Ca^{2+}]_i$ (Fig. 3*A*), and PDBu does not increase capacitative $Ca^{2+}$ influx in these cells (Y. M. Usachev and S. A. Thayer, unpublished observations). Indeed, activation of PKC generally inhibits capacitative $Ca^{2+}$ influx (Venkatachalam et al., 2004). The effect of PKC on $Ca^{2+}$ sequestration in the ER was more dramatic in the protocols that measured $[Ca^{2+}]_{ER}$ directly (Fig. 4*B*,*D*) relative to protocols that examined $[Ca^{2+}]_i$ recovery kinetics in PMCA4-depleted cells (Fig. 2). This quantitative difference can be explained in part by expression in DRG neurons of another PMCA isoform, PMCA2 (Usachev et al., 2002). This isoform is not stimulated by PKC (Enyedi et al., 1997). Thus, the effect of accelerated SERCA function on $[Ca^{2+}]_i$ recovery was diluted by the presence of PMCA2 (Fig. 2). Additionally, the stores were filled with $Ca^{2+}$ by repeated electrical stimulation in the indo-1 recordings (Fig. 2), whereas the rate of $Ca^{2+}$ uptake into the empty stores was assayed in the Mag-indo-1 experiments (Fig. 4). SERCAs are known to be affected by luminal $Ca^{2+}$ in various cell types (John et al., 1998; Mogami et al., 1998; Gyorke et al., 2002; Li and Camacho, 2004), including DRG neurons (Usachev and Thayer, 1999). In summary, the PKC-mediated increase in $Ca^{2+}$ clearance rate corresponded to an increased uptake rate as indicated by complimentary measurements of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ER}$.

The mechanism by which PKC activation leads to accelerated $Ca^{2+}$ uptake in DRG neurons is unclear. The results presented in Figure 4*F* suggest that PKC is stimulating the $Ca^{2+}$ pump rather than inhibiting the leak of $Ca^{2+}$ from the ER. Data on the regulation of SERCA-type $Ca^{2+}$ pumps by direct phosphorylation are limited. Phosphorylation of SERCA2a and SERCA2b by CaMK, but not by PKC or PKA, was reported for cardiac and smooth muscle (Hawkins et al., 1994; Allen and Katz, 1996; Grover et al., 1996). However, a more common mechanism is phosphorylation of accessory proteins such as phospholamban in cardiac and slow-twitch skeletal muscle (Tada and Toyofuku, 1998; MacLennan and Kranias, 2003). PKA- or CaMK-mediated phosphorylation of phospholamban reduces the inhibitory interaction of the protein with SERCA2a, resulting in stimulated pump and increased contractility. Phospholamban is also phosphorylated by

PKC, although the physiological outcome of direct phosphorylation by this kinase is unclear (Allen and Katz, 1996). Activation of PKC-$\alpha$ removes inhibition of protein phosphatase-1, leading to dephosphorylation of phospholamban and inhibition of SERCA2 in heart muscle (Braz et al., 2004). Although phospholamban has not been detected in neurons (Plessers et al., 1991), the presence of an analogous protein in brain has been suggested (Dou and Joseph, 1996). Alternatively, phosphorylation of a cytoplasmic domain on calnexin, an ER transmembrane protein that acts to chaperone protein folding within the ER, alters SERCA2b activity when overexpressed in *Xenopus* oocytes (Roderick et al., 2000). Calnexin and SERCA isoform 2b are ubiquitously expressed in brain (Krijnse-Locker et al., 1995; Baba-Aissa et al., 1998), making it likely that calnexin–SERCA2b interaction is involved in shaping $Ca^{2+}$ signals in neurons. Another ER chaperone protein, calreticulin, can also modulate SERCA2b activity, although it is not clear whether calreticulin is regulated by phosphorylation (John et al., 1998; Li and Camacho, 2004). Future studies will determine the target of PKC phosphorylation that is responsible for the increased $Ca^{2+}$ sequestration described here.

In sensory neurons, activation of PKC orchestrates a coordinated reduction in cytoplasmic $Ca^{2+}$ levels by inhibiting influx (Rane and Dunlap, 1986; Gross and MacDonald, 1989; Boland et al., 1991; Diversepierluissi and Dunlap, 1993), facilitating $Ca^{2+}$ efflux (Usachev et al., 2002), and, as shown here, accelerating $Ca^{2+}$ sequestration. Such coordinated modulation of the $Ca^{2+}$ signal resembles phosphorylation-dependent processes in cardiac muscle, in which changes in the levels of cAMP triggered by activation of $\beta$ adrenergic receptors regulate $Ca^{2+}$ influx, uptake, and release to produce coordinated chronotropic and inotropic responses (Petrashevskaya et al., 2002). We suggest that this type of coordinated modulation of $Ca^{2+}$ signaling processes may prove common in neurons.

Accelerated $Ca^{2+}$ clearance may affect many functions in sensory neurons, including excitability, secretion, gene expression, and even survival. An increased rate of $[Ca^{2+}]_i$ recovery reduces prominent $Ca^{2+}$-activated $K^+$ currents in DRG neurons (Sah, 1996). The resulting attenuation of the slow afterhyperpolarization in these cells is predicted to reduce spike frequency adaptation and to increase excitability (Abdulla and Smith, 1997; Cordoba-Rodriguez et al., 1999; Bahia et al., 2005). The shape of the presynaptic $[Ca^{2+}]_i$ transient has profound effects on neurotransmitter release as does the level of residual $Ca^{2+}$ (Kamiya and Zucker, 1994; Chen and Regehr, 1999; Muschol and Salzberg, 2000; Korogod et al., 2005). Thus, accelerated $[Ca^{2+}]_i$ clearance would be expected to reduce the duration of the secretory response and improve the fidelity of high-frequency synaptic transmission (Talbot et al., 2003). A more rapid refilling of the ER with $Ca^{2+}$ will also affect $Ca^{2+}$ release from ryanodine- and $IP_3$-sensitive $Ca^{2+}$ stores (Berridge, 1998), as well as the store-operated $Ca^{2+}$ influx present in DRG neurons (Usachev and Thayer, 1999). An increase in SERCA-mediated $Ca^{2+}$ uptake could influence neurotoxic processes. Accelerating $[Ca^{2+}]_i$ clearance might protect from $Ca^{2+}$ overload (Nicholls and Budd, 2000; Orrenius et al., 2003; Verkhratsky and Toescu, 2003). Alternatively, full ER $Ca^{2+}$ stores are a prerequisite for some apoptotic pathways, so accelerated refilling of the ER could exacerbate toxic processes (Szabadkai and Rizzuto, 2004). Finally, activation of $Ca^{2+}$-dependent transcription factors is sensitive to the amplitude and duration of transient increases in $[Ca^{2+}]_i$ (Berridge, 1997; Bito et al., 1997; Fields et al., 2001), and, thus, enhanced sequestration could affect gene expression.

In summary, we found that activation of PKC accelerated SERCA-mediated $Ca^{2+}$ uptake into the ER. Kinase-dependent modulation of SERCA function, which has not been described previously for neurons, may influence a number of ER functions, including protein processing, apoptosis, and $Ca^{2+}$ signaling. The coordinated stimulation of $Ca^{2+}$ sequestration and extrusion mechanisms enables PKC to tune $[Ca^{2+}]_i$ responses to the changing signaling needs of the cell.

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