

CaMKII inactivation by extracellular Ca^{2+} depletion in dorsal root ganglion neurons

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Abstract

A mechanism by which Ca^{2+} /CaM-dependent protein kinase (CaMKII) is autophosphorylated by changes in *extracellular* calcium in the absence of detectable changes in cytoplasmic $[\text{Ca}^{2+}]_i$ has been identified. We find that when the external Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) is lowered, Ca^{2+} is released from intracellular stores to maintain a constant cytoplasmic Ca^{2+} level, gradually depleting the endoplasmic Ca^{2+} stores. Accompanying the store-depletion is a rapid decrease in CaMKII activity. Approximately 25% of the measured CaMKII autophosphorylation in DRG neurons in culture can be regulated by Ca^{2+} flux from intracellular stores caused by manipulating $[\text{Ca}^{2+}]_o$, as shown by blocking refilling of store-operated Ca^{2+} -channels with SK&F 96365, Ruthenium Red, and a partial block with Ni^{2+} . Blocking voltage-gated Ca^{2+} -channels with either isradipine or SR 33805, had no effect on CaMKII autophosphorylation induced by restoring Ca^{2+}_o to normal after depleting the intracellular Ca^{2+} stores. These results show that removal of Ca^{2+}_o has profound effects on intracellular Ca^{2+} signaling and CaMKII autophosphorylation, in the absence of measurable changes in intracellular Ca^{2+} . These findings have wide-ranging significance, because $[\text{Ca}^{2+}]_o$ is manipulated in many experimental studies. Moreover, this explanation for the paradoxical changes in CaMKII phosphorylation in response to manipulating $[\text{Ca}^{2+}]_o$ provides a possible mechanism linking activity-dependent depletion of Ca^{2+} from the synaptic cleft to a protein kinase regulating many neuronal properties.

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1. Introduction

Ca^{2+} /CaM-dependent protein kinase (CaMKII), is a highly abundant enzyme present in neurons, which can function as a molecular switch converting intracellular Ca^{2+} (Ca^{2+}_i) signals into short-, intermediate-, and long-term responses through phosphorylation of diverse substrates involved in exocytosis, transcriptional, and translational processes [1]. Curiously, several studies have reported that CaMKII autophosphorylation is also regulated by changes in the concentration of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) [2–6], but the mechanism is unknown. In several experimental systems, basal levels of the phosphorylated, autonomously active form

of CaMKII are quite high, but lowering the $[\text{Ca}^{2+}]_o$ reduces CaMKII autophosphorylation dramatically. The initial explanation was that this effect was secondary to a reduction in endogenous synaptic activity under low Ca^{2+}_o conditions. However, studies reporting the same phenomenon in cultured dorsal root ganglion neurons (DRG) lacking spontaneous activity and synapses in culture indicate that this cannot be the only mechanism [5].

Here we provide evidence that CaMKII autophosphorylation regulated by $[\text{Ca}^{2+}]_o$ is not affected by blocking a wide range of voltage-gated Ca^{2+} -channels (VGCC), but that Ca^{2+} -flux from intracellular stores in response to changes in $[\text{Ca}^{2+}]_o$ regulates CaMKII autophosphorylation. Manipulations that decrease stores, e.g. removing Ca^{2+}_o , do not affect bulk $[\text{Ca}^{2+}]_i$ but act to rapidly decrease CaMKII phosphorylation. Additionally, re-addition of Ca^{2+}_o modestly

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increases resting $[Ca^{2+}]_i$ and strongly activates CaMKII. Thus, this signaling pathway permits $[Ca^{2+}]_O$ to directly regulate the activation state of CaMKII independent of neuronal depolarization and neurotransmitter-mediated activity. Rapid dephosphorylation of CaMKII over several minutes is within a physiological time frame. $[Ca^{2+}]_O$ -mediated regulation of Ca^{2+}_i signaling involving CaMKII may underlie an overlooked but important mechanism contributing to synaptic plasticity. In order to address this issue we further studied $[Ca^{2+}]_O$ -dependent regulation of CaMKII in DRG neurons.

2. Materials and methods

2.1. Cell culture

Campanot chambers were made of Teflon and attached to collagen-coated 35 mm culture dishes as described in Ref. [7]. DRG neurons were dissociated from 13.5-day old mouse embryos and plated at a density of 0.5×10^6 cells/ml into each side compartment in Eagle MEM with Earle's salts used for culturing neurons containing 5% horse serum and 100 ng/ml nerve growth factor as described previously [8]. For imaging studies, DRG neurons were plated at a density of 0.25×10^6 cells/ml onto glass coverslips. Non-neuronal cell division was inhibited by the addition of 13 μ g/ml fluoro-2-deoxyuridine and uracyl 1 day following plating for 4–5 days. Cultures were subsequently used for experiments 3–4 weeks after plating at which time they display a mature axonal outgrowth.

2.2. Drugs

The following drugs were used: isradipine (Alomone Labs, Jerusalem, Israel), flunarizine, ionomycin, ω -agatoxin-IVA, and ω -conotoxin-GVIA (EMD Biosciences Inc., San Diego, CA), Fluo-4/AM and Indo-1/AM (Molecular Probes, Eugene OR), caffeine, EGTA, gadolinium chloride, lanthanum chloride, nickel chloride, and nifedipine (Sigma, St. Louis, MO), KB-R7943, MRS 1845, Ruthenium Red (RR), SK&F 96365, and SR 33805 (Tocris Cookson, Ellisville, MO). In cases where drugs were dissolved in DMSO, final concentrations did not exceed 0.1% except for MRS 1845 where the DMSO concentration was 0.16% which was included in controls.

2.3. Autophosphorylation of CaMKII at $^{286/287}$ Thr

CaMKII autophosphorylation at $^{286/287}$ Thr was analyzed by immunoblotting using a phosphorylation-site specific antibody that recognizes CaMKII only when it is autophosphorylated at Thr-286 (α) or Thr-287 (β , γ , δ) [9]. Neurons were incubated overnight in serum- and growth factor-free medium. The following day, the medium was exchanged four times with a physiological saline solution containing 50 nM free Ca^{2+}_O (referred to in the figures as 0 Ca^{2+}_O) [4]. This was

obtained by substituting (in mM): 0.76 $CaCl_2$, 1.13 $MgCl_2$, and 2 EGTA in a HEPES-buffered saline (pH 7.38) containing 10 mM glucose. Free Ca^{2+} concentrations were calculated using Maxchelator [10]; EGTA was omitted in experiments with Gd^{3+} , La^{3+} , and Ni^{2+} as it binds these cations with much higher affinity than Ca^{2+} . Following incubation in 50 nM free Ca^{2+}_O for 60 min, the $[Ca^{2+}]_O$ was raised by rapidly exchanging the media within the compartment with media containing 1.8 mM free Ca^{2+}_O and incubating for 45 s (1.8 Ca^{2+}). (Molloy and Kennedy [2] observed that CaMKII activity decreases to a stable baseline by 30 min in 0 Ca^{2+}_O .) Treated neurons were subsequently lysed in 100 μ l $2\times$ sample buffer for electrophoresis and immunoblot analysis.

For quantification of $^{286/287}$ Thr autophosphorylation, 15 μ l of lysate from control and treated neurons were resolved in parallel by SDS-PAGE in duplicate 10% NOVEX Bis-Tris gels (Invitrogen, Carlsbad, CA) and electroblotted to PVDF membranes (Immobilon-P, from Millipore, Bedford, MA). Membranes were blocked in 5% non-fat dry milk in TBS-T for 2 h at room temperature, washed, and incubated in antibody that recognized total CaMKII at 1:2500 (mAb 38, from BD Biosciences Pharmingen, San Diego, CA) or phosphosite-specific antibody (1:10,000, from Dr. Y. Yamagata, Laboratory of Neurochemistry, Okazaki, Japan) overnight at 4 °C. Incubated membranes were washed and incubated with HRP-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at room temperature. The immunocomplexes were visualized with ECL Plus substrate (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified with ImageQuant and Storm image analysis system (Molecular Dynamics, Sunnyvale, CA). Relative autophosphorylation at $^{286/287}$ Thr was compared by normalization of the RFU obtained with the phosphorylated enzyme to that of the total enzyme in the same sample from parallel experiments.

2.4. Immunocytochemistry

DRG neurons were fixed in 4% paraformaldehyde containing 10 mM EGTA and 4% sucrose. Following fixation, neurons were permeabilized with 0.3% Triton X-100 for 5 min and free aldehydes were quenched with 50 mM ammonium chloride for 10 min. After blocking in 3% NGS, primary antibodies were incubated overnight at 4 °C. The following day, neurons were incubated for 2 h in secondary antibodies and mounted in Vectashield (Vector Labs, Burlingame, CA). The antibodies and dilutions used were: anti-phospho-CaMKII (1:500, Upstate USA Inc., Charlottesville, VA), anti-CaMKII (1:500, mAb 38, BD Biosciences Pharmingen), Alexa 488 goat anti-rabbit (1:500; Molecular Probes). All antibodies were diluted in 3% NGS.

2.5. Intracellular Ca^{2+} measurements

Caffeine-evoked Ca^{2+} transients in DRG neurons were measured using a Bio-Rad (Hercules, CA) 1024 visible/UV

confocal microscope and a Nikon 40× 1.3 numerical aperture oil immersion objective on a Nikon inverted microscope. Quantitative Ca^{2+} measurements were made using ratiometric measurements of fluorescence intensity at 460 and 405 nm emission from DRG neurons loaded by incubation in 7.5 μM indo-1/AM and excited by an argon-ion laser at 350 nm [53]. Two-photon Ca^{2+} imaging with Fluo-4/AM were measured using a Bio-Rad Radiance 2100 MP confocal microscope. Relative changes in Ca^{2+} were made using measurements of fluorescence intensity from DRG neurons loaded by incubation in 9.1 μM Fluo-4/AM and excited by a Mira 900 tuned to 800 nm. Measurements were performed at room temperature in HEPES-buffered balanced salt solution (pH 7.38). In-cell calibration, as described in [54], was used to provide an estimate of the $[\text{Ca}^{2+}]_i$ associated with the fluorescence ratios. Briefly, R_{\min} and R_{\max} were determined in neurons permeabilized by 10 μM ion-

omycin, in solutions containing 1.8 mM Ca^{2+} and 0 mM Ca^{2+} /10 mM EGTA, under the same intensifier gain and pin-hole settings that were used during the experiments. Measurements of $[\text{Ca}^{2+}]_i$ were made within an optical plane passing through the center of the nucleus in the area of the cytoplasm midway between cell membrane and the nucleus. The area of measurement comprised $\sim 1/8$ of the area of cytoplasm in the plane of section. The measured responses were uniform within different regions of the cell on the time scale reported in these experiments. Normalized fluorescence values for Fluo-4 were calculated by first subtracting background and rationing to the fluorescence intensity immediately prior to re-addition of Ca^{2+}_o with the equation: $F = (F - F_0)/F_0$. To compensate for Fluo-4 photo-bleaching due to prolonged imaging, controls were performed where DRG neurons were imaged in 1.8 mM Ca^{2+}_o -containing saline.

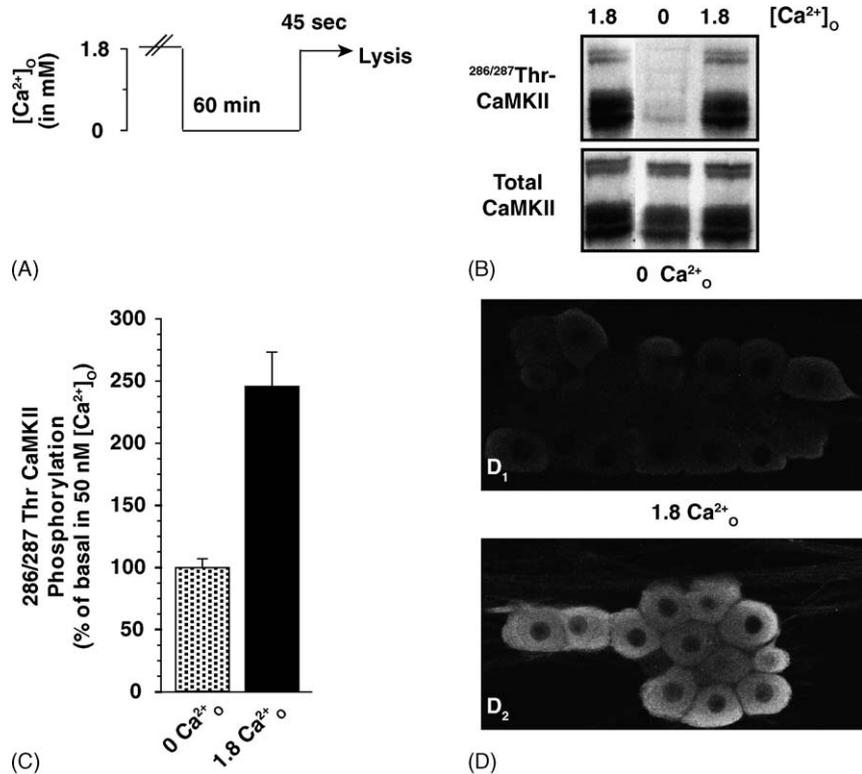


Fig. 1. DRG neurons exhibit high basal CaMKII autophosphorylation at $^{286/287}\text{Thr}$ that is regulated by Ca^{2+}_o . (A) In the Ca^{2+}_o -stimulation paradigm, DRG neurons were incubated in culture medium containing 50 nM free Ca^{2+} (0 Ca^{2+}_o) for 60 min followed by a 45 s treatment with 1.8 mM Ca^{2+}_o (1.8 Ca^{2+}_o). (B) Resting neurons in a medium containing 1.8 mM Ca^{2+}_o display strong CaMKII phosphorylation at $^{286/287}\text{Thr}$. Incubation for 60 min in 50 nM Ca^{2+}_o decreased the level of CaMKII phosphorylation; re-addition of extracellular calcium rapidly activated CaMKII. (C) Stimulation of DRG neurons with 1.8 mM Ca^{2+}_o increased phosphorylation at $^{286/287}\text{Thr-CaMKII}$ by several-fold. Relative autophosphorylation of CaMKII at $^{286/287}\text{Thr}$ was calculated by normalizing the relative immunoreactivity (RFU) of the phosphorylated sample to that of the total enzyme present in the same sample. Data are expressed as mean \pm S.E.M. of percentage phosphorylation normalized to basal levels in 50 nM Ca^{2+}_o ($n=3$). (D) DRG neurons were incubated in 0 Ca^{2+}_o . Ca^{2+}_o -stimulation was terminated by fixation in 4% paraformaldehyde containing 10 mM EGTA to block fixation-dependent Ca^{2+} influx [50,51]. Weak immunoreactivity for $^{286/287}\text{Thr-CaMKII}$ is observed in unstimulated neurons and entirely absent from axons (D_1 and D_2). Re-addition of 1.8 mM Ca^{2+}_o activates CaMKII as seen by strong immunoreactivity for $^{286/287}\text{Thr-CaMKII}$ in both the cell bodies and axons (D_2). The boxed regions indicate the field of higher magnification. There appears to be an increase in staining in both the cytoplasmic and nuclear membrane compared to the intracellular compartment. Staining is predominately excluded from the nucleus.

3. Results

3.1. Ca^{2+}_o regulates CaMKII phosphorylation

We observed that the level of autophosphorylated CaMKII was dependent on the concentration of Ca^{2+}_o (Fig. 1). Removing Ca^{2+}_o for 60 min decreased CaMKII phosphorylation to very low levels (Fig. 1B and D). Re-addition of Ca^{2+}_o rapidly restored phosphorylation to previous levels (Fig. 1B and C); phosphorylation at $^{286/287}Thr$ increased by $245 \pm 27.6\%$ relative to basal in 50 nM Ca^{2+}_o ($n=3$). The increase in phosphorylation mediated by Ca^{2+}_o is much greater than that mediated by activity-evoked changes, e.g. action potentials, in $[Ca^{2+}]_i$, which result in $\sim 25\%$ increase in CaMKII autophosphorylation and Ca^{2+} -independent activity [5]. By immunocytochemistry, we found weak staining for $^{286/287}Thr$ -CaMKII in DRG cell bodies incubated in low Ca^{2+}_o ; immunoreactivity was entirely absent from axons (Fig. 1D₁). Stimulation by 1.8 mM Ca^{2+}_o activated CaMKII as seen by strong immunoreactivity for $^{286/287}Thr$ -CaMKII in both the cell bodies and axons (Fig. 1D₂). Staining was predominately excluded from the nucleus in both unstimulated and stimulated neurons (Fig. 1D).

3.2. Basal CaMKII phosphorylation decays rapidly in $0 Ca^{2+}_o$

We more thoroughly characterized the response of DRGs to changes in Ca^{2+}_o by examining both the time course of CaMKII phosphorylation due to increasing time in $0 Ca^{2+}_o$ as well as sensitivity to increasing $[Ca^{2+}]_o$. CaMKII autophosphorylation at $^{286/287}Thr$ rapidly decreased with increasing time in low Ca^{2+}_o (Fig. 2A) [2–6]. We observed that phosphorylation increased in a dose-dependent manner; half-maximal activation of CaMKII occurred at 2.6 mM Ca^{2+}_o (Fig. 2B). Despite intense research in this ubiquitous protein kinase, the mechanism regulating CaMKII autophosphorylation in response to changes in Ca^{2+}_o have remained obscure for many years.

3.3. Intracellular Ca^{2+}_i does not change during incubation in $0 Ca^{2+}_o$

Confirming previous results [5], two-photon Ca^{2+} -imaging with Fluo-4 showed that decreasing Ca^{2+}_o produced no measurable changes in bulk $[Ca^{2+}]_i$ over 60 min (Fig. 3). Previously, Eshete and Fields [5] did not observe an increase in $[Ca^{2+}]_i$ with re-addition of Ca^{2+}_o . We utilized two-photon Ca^{2+} - Ca^{2+} -imaging with Fluo-4 (Fluo-4 is more sensitive to $\Delta[Ca^{2+}]_i$ as compared with Indo-1) to minimize both photo-bleaching and cellular damage due to prolonged imaging. We were able to detect a small increase in $[Ca^{2+}]_i$ following re-addition of Ca^{2+}_o (Fig. 3, inset). For comparison, application of 10 mM caffeine elicited a large, rapid increase in $[Ca^{2+}]_i$ (11.5-fold higher compared

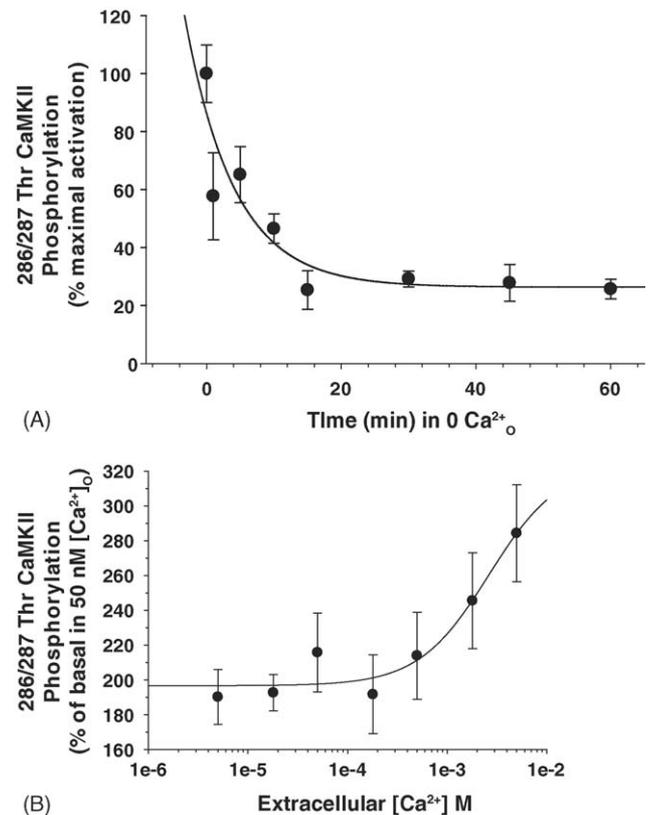


Fig. 2. Ca^{2+}_o -regulation of CaMKII phosphorylation decreases rapidly and is dose-dependently activated by Ca^{2+}_o . (A) Phosphorylation of CaMKII at $^{286/287}Thr$ rapidly decreased with increasing incubation in 50 nM free Ca^{2+} . DRG neurons were incubated in low Ca^{2+} for different time periods. The minimum time to transfer cells from normal Ca^{2+} to low Ca^{2+} is 1 min. Data were fit to three-parameter exponential decay to obtain the decay rate constant, $0.137 s^{-1}$. CaMKII phosphorylation relative to control was decreased to less than 50% by 7 min in 50 nM free Ca^{2+} ($n=4$). (B) DRG neurons were incubated in a medium containing 50 nM free Ca^{2+} for 60 min and then stimulated for 45 s with increasing $[Ca^{2+}]_o$. As in (Fig. 1(C)), relative autophosphorylation of CaMKII at $^{286/287}Thr$ was compared for each condition by normalizing the RFU of the phosphorylated sample to that of the total enzyme present in the same sample. The ratio of phospho:total CaMKII for each $[Ca^{2+}]_o$ was then normalized to stimulation by 50 nM free Ca^{2+} ; data were fit to a four-parameter logistic equation to obtain an EC_{50} of 2.6 mM as mean \pm S.E.M. ($n=3$).

with Ca^{2+}_o -stimulation) that decayed to basal levels over time.

3.4. Caffeine-sensitive stores are depleted during incubation in $0 [Ca^{2+}]_o$

We suspected that microdomains of intracellular Ca^{2+} might be changing in response to Ca^{2+}_o manipulation, but that they were undetectable with standard confocal Ca^{2+} imaging. One possibility was that if Ca^{2+} were leaking across the membrane under low Ca^{2+}_o conditions, it might be replenishable from Ca^{2+} supplied from intracellular stores to maintain the $[Ca^{2+}]_i$ at a physiologically stable level. We tested the hypothesis that Ca^{2+} may be released from intracel-

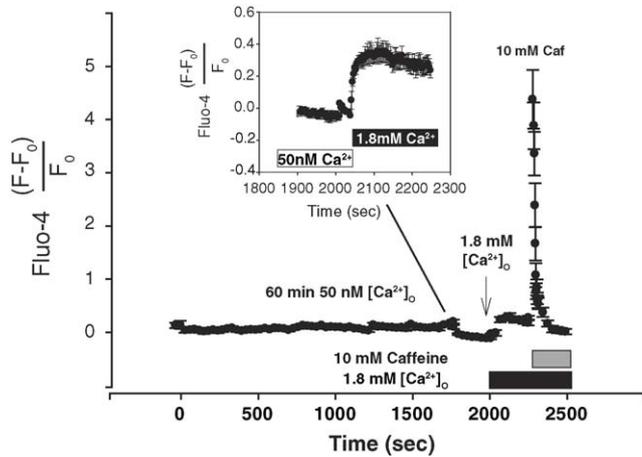
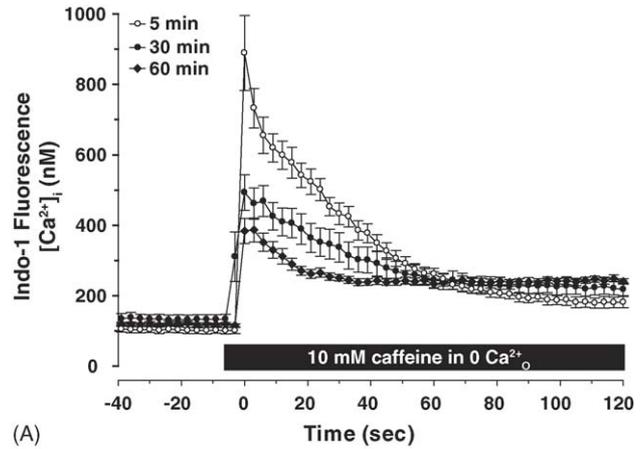


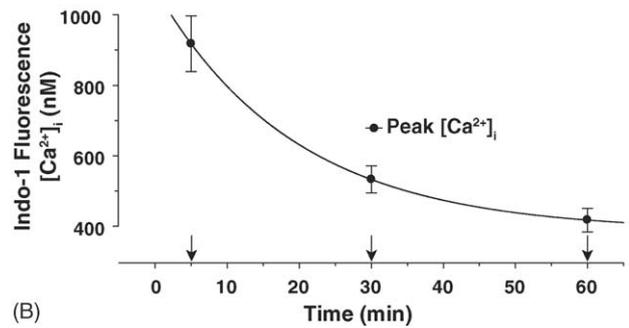
Fig. 3. Incubation in 0Ca^{2+}_o does not alter intracellular Ca^{2+} . Incubation of DRG neurons in 0Ca^{2+}_o did not decrease intracellular calcium measured by Fluo-4. DRG neurons were imaged at 30 s intervals in $50\text{nM}\text{Ca}^{2+}_o$ for 65 min followed by re-addition of $1.8\text{mM}\text{Ca}^{2+}_o$. Control neurons were incubated in $1.8\text{mM}\text{Ca}^{2+}_o$ and imaged to adjust for photo-bleaching under constant Ca^{2+} levels. Bleaching was not adjusted for later time points, e.g. prior to re-addition of Ca^{2+}_o as the acquisition rate was changed to 3 s intervals. Fluorescence values were calculated by subtracting background and normalizing to the fluorescence (F_0) immediately prior to $1.8\text{mM}\text{Ca}^{2+}_o$. Re-addition of Ca^{2+}_o rapidly increased Ca^{2+}_i ; to a new baseline. 10mM caffeine rapidly increased Ca^{2+}_i ; that decayed to baseline. Fluorescence values are the mean \pm S.E.M. of 14 cells. *Inset*: larger time resolution demonstrates that re-addition of $1.8\text{mM}\text{Ca}^{2+}_o$ rapidly increases Ca^{2+}_i . The initial decrease in fluorescence is due to photo-bleaching of the dye. Compared to the effects of 10mM caffeine, the relative changes in Ca^{2+}_i corresponding to Ca^{2+}_o are small but not negligible.

lular stores in DRG neurons incubated in reduced $[\text{Ca}^{2+}]_o$, by measuring the release of Ca^{2+} from caffeine-sensitive stores using ratiometric Indo-1 confocal microscopy, in DRG neurons incubated in reduced external $[\text{Ca}^{2+}]_o$ for 5–60 min.

We found that although there were no measurable changes in resting $[\text{Ca}^{2+}]_i$ with prolonged incubation in 0Ca^{2+}_o ; for up to 60 min, the peak amplitude of the Ca^{2+}_i transient induced by 10mM caffeine decreased the longer DRG neurons were incubated in low external Ca^{2+} concentrations (Fig. 4A and B). Accompanying this decline in peak Ca^{2+} response, the caffeine-induced Ca^{2+}_i -transient decayed more rapidly, consistent with the hypothesis that the stores were becoming depleted of Ca^{2+} and less able to sustain release (Fig. 4A). These two effects are consistent with a partial depletion of Ca^{2+}_i stores in reduced extracellular $[\text{Ca}^{2+}]_o$. Comparison of the CaMKII phosphorylation level over time in reduced Ca^{2+}_o (Fig. 2A) and the decline in the peak Ca^{2+}_i response to caffeine stimulation (Fig. 4B) showed that the decrease in Ca^{2+}_i response and CaMKII phosphorylation followed a similar time course and were well correlated (Fig. 5 and inset). The extent of store-depletion depletion may act to regulate the basal autophosphorylation state of CaMKII. We therefore tested the hypothesis that Ca^{2+} entry through SOC contributes to CaMKII activation.



(A)



(B)

Fig. 4. Increasing incubation time in 0Ca^{2+}_o depletes caffeine-sensitive intracellular Ca^{2+} stores. (A) Brief exposure of DRG neurons to 10mM caffeine rapidly elicits Ca^{2+}_i transients that slowly decayed over tens of seconds. Increasing the incubation time in 0Ca^{2+}_o increases the decay rate of the caffeine-induced Ca^{2+}_i -transient. Baseline $[\text{Ca}^{2+}]_i$ was measured until stable and then 10mM caffeine was applied. Raw fluorescence data obtained from selected ROI was converted to $[\text{Ca}^{2+}]_i$ using the following equation: $[\text{Ca}^{2+}]_i = K_d \times \beta(R - R_{\min}) / (R_{\max} - R)$ [52]. Data for Ca^{2+}_i transients were fit to a three-parameter exponential decay function to obtain decay rates for caffeine-induced transients; the decay rates were: 0.033 s^{-1} for 5 min ($n = 7$ cells), 0.050 s^{-1} for 30 min ($n = 7$ cells), and 0.095 s^{-1} for 60 min ($n = 8$ cells) (one-way ANOVA testing the decay rate for 5, 30, and 60 min incubation in 0Ca^{2+}_o : $F_{2,21} = 16.31$, $p < 0.001$). Although incubation in 0Ca^{2+}_o for 30 min did not significantly increase the decay rate over 5 min, by 60 min, the decay rate was significantly increased. (B) The peak Ca^{2+}_i response decreased with longer incubation in 0Ca^{2+}_o with a decay rate of 0.058 s^{-1} (one-way ANOVA testing peak $[\text{Ca}^{2+}]_i$ at 5, 30, and 60 min: $F_{2,21} = 24.65$, $p < 0.001$). Baseline $[\text{Ca}^{2+}]_i$ prior to caffeine treatment was not significantly affected with prolonged incubation in 0Ca^{2+}_o ($p \gg 0.05$).

3.5. Store-operated Ca^{2+} -permeable channels contribute to CaMKII activation by Ca^{2+}_o

DRG neurons express all of the components that underlie capacitative Ca^{2+} -entry (CCE) and Ca^{2+} -induced Ca^{2+} release (CICR) commonly observed in non-excitable cells [11–14]. Both RyR- and IP₃R-sensitive Ca^{2+} stores have been demonstrated to be functionally coupled in DRG neurons and contribute to intracellular Ca^{2+} signaling [15]. Additionally, DRG neurons express a full complement of non-selective cation channels that form the transient receptor potential channel family (TRP), which responds to stimuli such as cold,

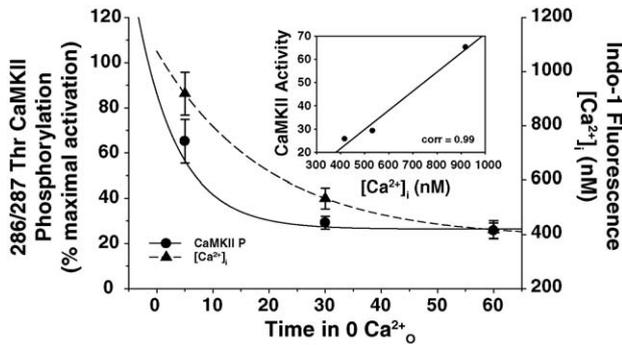


Fig. 5. The decay in CaMKII phosphorylation correlates with the decrease in the peak Ca^{2+} ; response. Data presented in Fig. 2A and Fig. 4B were compared to demonstrate that CaMKII phosphorylation (●) decays proportionally to the decrease in the peak Ca^{2+} ; transient (▲) produced by 10 mM caffeine. *Inset*: scatter plot of CaMKII activity and peak Ca^{2+} ; demonstrate that both functions are well correlated (correlation value = 0.99).

heat, and stretch. Depletion of intracellular stores and subsequent activation of these channels would provide a route of Ca^{2+} entry in resting neurons, independent of VGCC-mediated Ca^{2+} influx.

DRG neurons were incubated in 50 nM free Ca^{2+} in the presence of inhibitors of store-operated influx. After 60 min, the concentration of Ca^{2+} ₒ was increased to 1.8 mM. Ruthenium Red and SK&F 96365, which are blockers of non-voltage gated Ca^{2+} -permeable channels, including store-operated channels (SOC), inhibited the activation of CaMKII significantly. Although not statistically significant, Ni^{2+} modestly decreased CaMKII phosphorylation by $8 \pm 6\%$ ($n = 10$; Fig. 6). (Note that Ni^{2+} occludes Ca^{2+} efflux from DRG neurons [12], therefore DRG neurons were first incubated in 0Ca^{2+} ₒ and then switched to a medium containing 1.8 mM Ca^{2+} ₒ in the presence of 500 μM Ni^{2+} .)

Other drugs that can block SOC were tested, but the results were inconclusive, possibly because of their known actions on other Ca^{2+} -permeable channels. 2-APB blocks several SOC and IP_3 -mediated Ca^{2+} release; however, 2-APB at increasing concentrations, activates TRPV-channels [16] so the effects of this drug are more difficult to interpret. We found that 100 μM 2-APB did not block CaMKII activation (Fig. 6). Flufenamic acid, both a broad inhibitor of non-selective cation channels and several TRP-channels, failed to block CaMKII activation (Fig. 6). However, there have been reports of the related compound niflumic acid releasing Ca^{2+} from ryanodine-sensitive stores that might act to reverse the affects on Ca^{2+} -influx [17]. We also tested several di- and trivalent cations; neither Gd^{3+} nor La^{3+} at 300 μM blocked activation of CaMKII. Mg^{2+} at 5 mM was ineffective (not shown).

We used Ca^{2+} imaging to test the alternative hypothesis that the inhibitory effect of SK&F 96365 on CaMKII was caused by blocking Ca^{2+} -influx that occurs following re-addition of 1.8 mM Ca^{2+} ₒ. DRG neurons were incubated in low Ca^{2+} ₒ for 65 min and then switched to saline containing 10 μM SK&F 96365. Normalized fluorescence increased

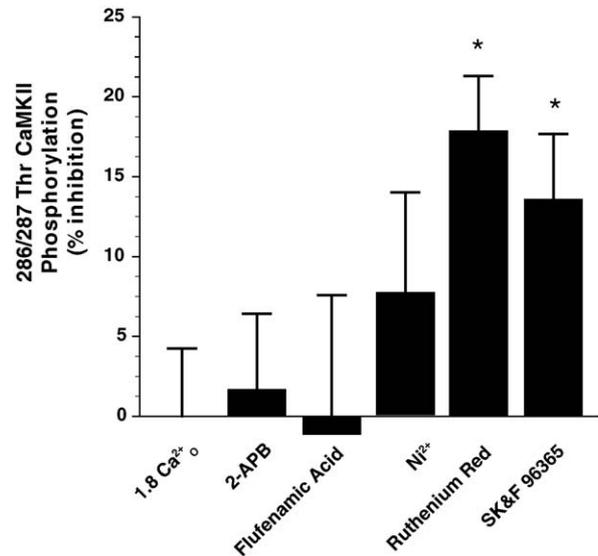


Fig. 6. CaMKII activation by Ca^{2+} ₒ is sensitive to inhibitors of SOCs. DRG neurons were incubated in SOC blockers prior to stimulation by 1.8 Ca^{2+} ₒ. Phosphorylation of CaMKII in 0Ca^{2+} ₒ was $11 \pm 2\%$ of maximal activation. Ruthenium Red (RR) at 100 μM and SK&F 96365 at 10 μM modestly inhibited the activation of CaMKII by $19 \pm 4\%$ ($n = 16$) and $14 \pm 4\%$, respectively ($n = 16$). 2-APB ($n = 12$) and flufenamic acid ($n = 9$) at 100 μM did not block CaMKII activation. Because EGTA effectively chelates Ni^{2+} , EGTA was excluded from the buffer. Following incubation in 0Ca^{2+} for 1 h in the absence of blockers, DRG neurons were then stimulated with 1.8 mM Ca^{2+} ₒ in the presence of Ni^{2+} (500 μM). Basal CaMKII phosphorylation in this nominally Ca^{2+} -free saline was $31 \pm 3\%$ of maximal phosphorylation by 1.8 Ca^{2+} ₒ ($n = 6$) which is higher than in experiments performed in the presence of EGTA to buffer $[\text{Ca}^{2+}]$ ₒ to low nM levels. Both RR and SK&F 96365 significantly decreased CaMKII activation (one-way ANOVA testing the effect of SOC blockers on Ca^{2+} ₒ stimulation: $F_{5,86} = 2.64$, $p = 0.029$).

following superfusion of the drug and reached a new plateau after several minutes (Fig. 7). SK&F 96365 did not block the increase in $[\text{Ca}^{2+}]$ ᵢ due to re-addition of Ca^{2+} ₒ. The decrease in Ca^{2+} -stimulated CaMKII, specifically by RR and SK&F 96365 suggest that components of the store-operated Ca^{2+} -influx pathway contribute to Ca^{2+} ₒ-mediated modulation of CaMKII.

3.6. Ca^{2+} ₒ-dependent activation of CaMKII in resting DRG neurons does not require influx through voltage-gated Ca^{2+} -channels

Ca^{2+} influx through voltage-gated Ca^{2+} -channels (VGCC) is the primary means of Ca^{2+} entry in excitable cells. However, these channels may also mediate Ca^{2+} -influx at rest, and the changes in Ca^{2+} in micro-domains near the membrane channels could couple to CaMKII activation and provide another mechanism for regulating CaMKII by $[\text{Ca}^{2+}]$ ₒ. We further tested whether Ca^{2+} ₒ activated CaMKII through either direct modulation of VGCC or influx through these channels but found no evidence to support this hypothesis. DRG neurons were incubated in inhibitors of VGCC, isradipine and SR 33805. Neither compound at 10 μM had an effect on CaMKII activation (Fig. 8). We also tested a

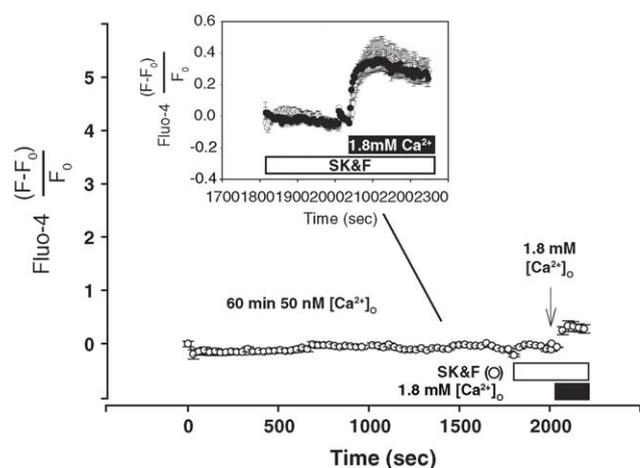


Fig. 7. SK&F 96365 does not affect Ca^{2+}_o -mediated influx. As in Fig. 1, incubation of DRG neurons in 50 nM free Ca^{2+}_o did not decrease $[\text{Ca}^{2+}]_i$. After incubating DRG neurons in 50 nM free Ca^{2+}_o for 60 min, neurons were incubated in 10 μM SK&F 96365 for 5 min prior to re-addition of 1.8 mM Ca^{2+}_o . Incubation in drug modestly increased Ca^{2+}_i above resting levels. Fluorescence values are the mean \pm S.E.M. of 13 cells. *Inset*: larger time resolution demonstrates that SK&F 96365 increased Ca^{2+}_i . Note that the increase in Ca^{2+}_i in the presence of drug (\circ) was indistinguishable from control (\bullet).

cocktail of Ca^{2+} -channel inhibitors at concentrations known to block activation of L-, N-, P/Q-, and T-type channels [18]. In the presence of the VGCC cocktail, we observed a modest $14 \pm 7\%$ ($n=3$) reduction in CaMKII activation which was not statistically significant. As a control, we tested whether reverse mode operation of the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger contributes to CaMKII activation [19,20]. However, KB-R7943 at 10 μM was inactive in modulating the CaMKII response to Ca^{2+}_o (Fig. 8). The dihydropyridine MRS 1845, which displays low μM affinity for both L-type channels and some

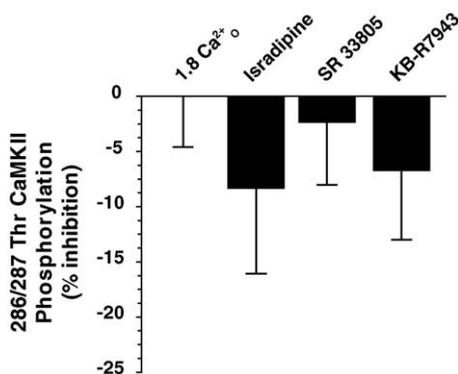


Fig. 8. Activation of CaMKII is independent of VGCC-mediated Ca^{2+} influx. Pre-incubation with either the L-type channel blockers isradipine or SR 33805 ($n=6$) at 10 μM failed to block CaMKII activation. Additionally, the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger inhibitor KB-R7943 at 10 μM ($n=6$) also did not affect activation of CaMKII by Ca^{2+}_o -stimulation. Basal phosphorylation at $^{286/287}\text{Thr}$ in 0 Ca^{2+}_o was $12 \pm 1\%$ of stimulation by 1.8 Ca^{2+}_o . Neither compound significantly affected Ca^{2+} -stimulation ($n=6$) suggesting that changes in $[\text{Ca}^{2+}]_o$ act at a site independent of VGCC.

SOC [21], did not significantly decrease Ca^{2+} -activation of CaMKII ($6 \pm 8\%$ decrease from control ($n=15$)).

4. Discussion

Reduction in $[\text{Ca}^{2+}]_o$ is a widely used experimental manipulation in studies of synaptic physiology and gap junction coupling, and the $[\text{Ca}^{2+}]_o$ can be depleted naturally under several physiological and pathophysiological conditions. We have found that changes in $[\text{Ca}^{2+}]_o$ act to regulate the autophosphorylation state of CaMKII through intracellular store-depletion and subsequent influx through non-voltage-gated channels. This provides a mechanism to link depletion of Ca^{2+}_o to intracellular signaling pathways to regulate cellular processes mediated by CaMKII. Activity-dependent changes in $[\text{Ca}^{2+}]_o$ thus may contribute to Ca^{2+} signaling through this novel mechanism to modulate intracellular stores and continuously tune CaMKII activity based on the extracellular environment. In this context, Ca^{2+}_o can function as an activity-dependent signaling molecule.

Many laboratories working in varied systems have demonstrated that free Ca^{2+} in the extrasynaptic space is regulated during trains of synaptic activity [22–26]. Additionally, mathematical modeling suggests that the degree of $[\text{Ca}^{2+}]_o$ depletion is highly frequency dependent [27]. In this context, action potential-induced fluctuations in $[\text{Ca}^{2+}]_o$ may act as an extracellular activity-dependent signal that modulates the balance between inactive and active CaMKII. However, it is less clear how depletion of $[\text{Ca}^{2+}]_o$ is linked to intracellular signaling. More recently, synaptic activity in addition to driving influx of Ca^{2+} through both VGCC and NMDA receptors also activates capacitative Ca^{2+} -entry (CCE) invoking both inositol 1,4,5-trisphosphate receptor (IP_3R)- and ryanodine receptor (RyR)-sensitive Ca^{2+} stores [28,29] to regulate presynaptic release properties [30–32].

Non-voltage gated Ca^{2+} -influx pathways mediated by SOC (including TRP channels), originally identified in non-excitabile cells [33] are widely expressed in neurons and contribute to signaling via detection of various extracellular stimuli [34,35]. The experimental manipulation of removing Ca^{2+}_o should be used cautiously as it impacts on CaMKII signaling in neurons.

4.1. CaMKII de-phosphorylation is regulated by intracellular store-depletion

We found by ratiometric Indo-1 Ca^{2+} imaging that prolonged incubation in 0 Ca^{2+}_o reduces caffeine-sensitive stores without net change in bulk cytoplasmic $[\text{Ca}^{2+}]$ (Fig. 4A and B). The decrease in CaMKII phosphorylation is regulated by the filled state of the intracellular stores. We also observed that re-addition of Ca^{2+}_o produced a modest increase in Ca^{2+}_i that was insensitive to SK&F 96365, a compound that partially decreases CaMKII activation by Ca^{2+}_o and occludes Ca^{2+}_i transients induced by intracellular store release. As

intracellular stores are progressively depleted, a signal is generated that activates SOC and the Ca^{2+} -release activated current (I_{CRAC}). The pharmacology for these channels has not been clearly worked out as they comprise a large family that may form heterodimeric channels with greatly different pharmacologies and properties dependent upon the cell type [36]. They are also regulated to some extent by $[\text{Ca}^{2+}]_{\text{O}}$ in the physiological range of synaptic cleft Ca^{2+} [33,37,38]. As observed in non-neuronal cells, re-addition of Ca^{2+} activates CCE through these SOC and then regulates downstream Ca^{2+} -signaling pathways. We have found that RR and SK&F 96365, compounds that block store-mediated influx, partially inhibit the activation of CaMKII (Figs. 6 and 7).

4.2. Store-operated channels may underlie an important influx mechanism regulating CaMKII

Our observations on the role of $\text{Ca}^{2+}_{\text{O}}$ in regulating $\text{Ca}^{2+}_{\text{i}}$ -dependent processes strongly argue that activity-dependent alterations in $\text{Ca}^{2+}_{\text{O}}$ directly regulate intracellular Ca^{2+} -dependent processes. We have observed that removal of $\text{Ca}^{2+}_{\text{O}}$ depletes ryanodine-sensitive stores (both IP_3R and RyR stores are coupled in DRG neurons [15]). The store-depletion paradigm that we used in these experiments required a prolonged incubation (30–60 min) in $0\text{Ca}^{2+}_{\text{O}}$; however, both the decrease in peak $\text{Ca}^{2+}_{\text{i}}$ and CaMKII phosphorylation occur within 10 min and plateau by 30 min (Figs. 2A, 4B and 5). The time course of CaMKII activation induced by re-addition of $\text{Ca}^{2+}_{\text{O}}$ is very rapid, occurring in less than 45 s [5].

Under physiological conditions, action-potential firing alone is sufficient to partially deplete intracellular stores [39]; however, activity itself activates voltage-gated Ca^{2+} -influx pathways. As DRG neurons are not spontaneously active, we utilized the $0\text{Ca}^{2+}_{\text{O}}$ paradigm to reproduce store-depletion independent of voltage-gated channel activation. We also observed that depletion of caffeine-sensitive stores decreased CaMKII phosphorylation. Influx of $\text{Ca}^{2+}_{\text{O}}$ may occur through a store-operated influx mechanism to act on CaMKII that is situated in subplasmalemmal domains or ER cistern that oppose the cell membrane [40,41]. As well, re-addition of $\text{Ca}^{2+}_{\text{O}}$ increases $\text{Ca}^{2+}_{\text{i}}$ to a much higher level directly beneath the membrane than within the cytoplasm [42]. Other experimental systems have demonstrated that intracellular stores act to regulate CaMKII activation [43] and CaMKII acts to potentiate SOC currents [37].

4.3. Additional mechanisms regulating CaMKII by $\text{Ca}^{2+}_{\text{O}}$

These studies do not rule out the possibility that additional mechanisms may contribute to the sensitivity of CaMKII to $\text{Ca}^{2+}_{\text{O}}$. In these experiments, however, blocking VGCC with either isradipine or SR 33805 did not block the increase in CaMKII phosphorylation upon restoring $\text{Ca}^{2+}_{\text{O}}$ to normal. Additional routes of Ca^{2+} influx that may contribute

to the remaining CaMKII phosphorylation may be due to other Ca^{2+} -permeable channels that are insensitive to RR and SK&F 96365. Smith et al. [26] recently reported an extracellular Ca^{2+} sensor that functions to regulate the excitability of presynaptic terminals via regulation of a non-selective cation channel. Although DRG neurons express a CaSR ([44] and unpublished observations), we did not observe activation of CaMKII with increasing $[\text{Gd}^{3+}]$, which activates the CaSR, arguing against its involvement in CaMKII regulation.

4.4. Implications for $\text{Ca}^{2+}_{\text{O}}$ -regulated CaMKII phosphorylation in synaptic plasticity

We found that activation of CaMKII was saturable with increasing $[\text{Ca}^{2+}]_{\text{O}}$ (Fig. 2B) and the EC_{50} for stimulation was within the range of Ca^{2+} that is observed within the synaptic cleft, making it highly sensitive to respond to changes in $\text{Ca}^{2+}_{\text{O}}$ that is modulated by synaptic activity [22,25,27]. Synaptic activity regulates intracellular Ca^{2+} stores through both IP_3 - and RyR -sensitive stores that exert effects on both short- and long-term plasticity [28,30,32,45–48] (see Berridge [49] for review). However, what is not clear is the degree to which $\text{Ca}^{2+}_{\text{O}}$ -modulation of intracellular signaling, e.g. CaMKII, contributes to the overall processes during periods of synaptic activity. However, as we and others have observed, a large amount of this basal autonomous activity is present in resting DRG neurons and highly dependent on $\text{Ca}^{2+}_{\text{O}}$. Manipulations that deplete intracellular stores act to decrease CaMKII activity. We have also observed that compounds that interfere with these intracellular stores, decrease CaMKII activation. Our observations on the role of $\text{Ca}^{2+}_{\text{O}}$ in regulating $\text{Ca}^{2+}_{\text{i}}$ -dependent processes strongly argue that activity-dependent alterations in $\text{Ca}^{2+}_{\text{O}}$ directly regulate intracellular Ca^{2+} -dependent processes that would have lasting effects on neuronal signaling.

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