Nucleoplasmic Calcium Is Required for Cell Proliferation*

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Ca2+ signals regulate cell proliferation, but the spatial and temporal specificity of these signals is unknown. Here we use selective buffers of nucleoplasmic or cytoplasmic Ca2+ to determine that cell proliferation depends upon Ca2+ signals within the nucleus rather than in the cytoplasm. Nuclear Ca2+ signals stimulate cell growth rather than inhibit apoptosis and specifically permit cells to advance through early prophase. Selective buffering of nuclear but not cytoplasmic Ca2+ signals also impairs growth of tumors in vivo. These findings reveal a major physiological and potential pathophysiological role for nucleoplasmic Ca2+ signals and suggest that this information can be used to design novel therapeutic strategies to regulate conditions of abnormal cell growth.

Ca2+ is a ubiquitous second messenger that mediates a wide range of cellular responses such as contraction, fluid and electrolyte secretion, exocytosis, gene transcription, and apoptosis (1). This ability to simultaneously control multiple processes occurs by careful modulation of Ca2+ signals, not only over time but in different subcellular regions as well (2). For example, polarized Ca2+ waves direct apical secretion in epithelia (3), whereas presynaptic increases in Ca2+ trigger neurotransmitter release (4) and mitochondrial increases in Ca2+ regulate apoptosis (5, 6). Ca2+ signals also can be regulated independently in the nucleoplasm relative to the cytoplasm (7, 8), but the physiological significance of this aspect of spatial control is not entirely understood. Nucleoplasmic Ca2+ signals have distinct effects on activation of transcription factors (9, 10) and kinases (11, 12), but it is not known whether nuclear Ca2+ signals also regulate more global aspects of cell function. Because cell proliferation (13, 14) and progression through the cell cycle (15, 16) are Ca2+-dependent, we investigated the relative roles of nuclear and cytoplasmic Ca2+ on cell growth.

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Cell Lines—SKHep1, HepG2, and HEK-293 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were used for all experiments. The cells were grown at 37 °C with 5% CO2:95% O2 in Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin-streptomycin and 10% heat-inactivated fetal bovine serum, all from Invitrogen. The cells were grown on glass coverslips overnight in the absence of serum before infection with each parvalbumin (PV)2 construct.

Generation of Parvalbumin Constructs and Adenoviral Infection— Constructs encoding red fluorescence protein (DsRed) from Clontech (Mountain View, CA) and targeted PV proteins (PV-NLS, PV-NES, and PV-NLS-CD) were PCR-amplified and subcloned into pShuttle-CMV (kindly provided by Bert Vogelstein, Johns Hopkins) by restriction digestion with XhoI and XbaI to generate pShuttle-CMV-PV-NLS-DSR, pShuttle-CMV-PVNES-DSR, and pShuttle-CMV-PVNLS-CD-DSR. Recombinant adenoviruses were generated by transformation of pShuttle-CMV-PV-NLS-DSR into AdEasier-1 cells, a derivative of BJ5183 bacteria already containing the adenovirus backbone plasmid pAdEasy-1. Positive recombinant adenoviruses (pAd-PV-NLS-DSR, etc.) were screened for resistance to kanamycin (Invitrogen) and restriction enzyme analysis using the PacI enzyme. Parvalbumin adenoviruses were amplified using HEK-293 cells and then purified (10, 17).

Measurement of BrdUrd Incorporation—Cell proliferation was measured by BrdUrd incorporation using an enzymelinked immunosorbent assay (Roche Applied Science) according to the manufacturer’s instructions. SKHep1 cells infected with parvalbumin constructs or HepG2 cells stably transduced with inducible parvalbumin constructs were plated 48 h after infection in 96-well culture plates (BD Biosciences, San Diego, CA) and treated for 2 h with BrdUrd labeling solution. The cells were then fixed, and anti-BrdUrd antibody was added. A colorimetric substrate was used, and BrdUrd incorporation was measured with a multiaplate reader.

Cell Counting Assay—SKHep1 cells (104 cells/ml) were plated on 35-mm dishes without serum and infected with targeted PV constructs. The cells were counted with trypan blue (Invitrogen) 24, 48, and 72 h after infection.

Apoptosis Assay—Two methods were used for measuring apoptosis. Caspase-3 enzyme activity was assayed using a caspase-3 activity kit with colorimetric detection (BD Biosciences) according to the manufacturer’s instructions. Alter-
natively, the cells were loaded with 0.2 μg/ml of the nuclear stain Hoechst 33258 (Molecular Probes, Eugene, OR), and then chromatin condensation and nuclear fragmentation were assessed by confocal microscopy. Apoptosis was induced using 500 nM staurosporine (Sigma–Aldrich) as a positive control.

**Cell Cycle Analysis**—Cells infected with targeted PV constructs were trypanosized, washed in phosphate-buffered saline (PBS), fixed overnight with ice-cold 70% EtOH at 4 °C, and then washed in staining buffer (Dulbecco’s PBS with 2% fetal calf serum and 0.01% NaN₃). After centrifugation the cells were supplemented with RNase (100 μg/ml) for 30 min at 37 °C and stained with propidium iodide (50 μg/ml) at room temperature in the dark. DNA content was determined using a FACSCalibur (BD Biosciences), and the data were analyzed using FlowJo software version 5.5.

**Immunoblotting**—Standard methods were used for immunoblots (8). Briefly, cells grown in 35-mm dishes were washed three times with PBS and solubilized in 80 μl of Nonidet P-40 containing a protease inhibitor mixture (Roche Applied Science). The protein concentration was determined spectrophotometrically, and 30 μg of protein was separated by electrophoresis in a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in PBST (PBS plus 0.1% Tween 20) for 20 min and then incubated with primary antibody (1:500). Commercially available antibodies for cyclin D1 (Stressgen, Victoria, Canada), cyclin E, B1, phospho-cdk1 and -cdk1 (BD Biosciences), p15, p21, p27, cdk4, phospho-cdc25c, and total cdc25c (Cell Signaling, Danvers, MA) and p-ERK, total ERK, ERK-5, total AKT, p-AKT, total CREB, and p-CREB (Cell Signaling) were used. Incubations were carried out overnight. After three washes with TBST, the membranes were incubated with peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (ECL plus; Amersham Biosciences), and the data were analyzed using FlowJo software version 5.5.

**Mitotic Index Measurements and Immunofluorescence**—The cells were labeled for phosphohistone-3 (Upstate Biotechnology, Inc., Chicago, IL) and γ-tubulin (Sigma–Aldrich) by immunofluorescence and then examined using a Zeiss LSM 510 confocal microscope (8, 12). Mitotic figures were scored for cells in each phase of mitosis according to the phospho-histone-3 distribution and DNA condensation (18, 19).

**Establishment of Inducible Tet-On HepG2 Cell Line**—The inducible Tet-On HepG2 cell line system was established using the PV-NLS and PV-NES constructs previously reported (20, 21), along with the Tet-On Gene expression system (Invitrogen).

**Animal Studies**—Male 4-week-old NCR nude mice (average body weight, 20 g) were obtained from Taconic Farms (Hudson, NY) and acclimated to laboratory conditions 1 week before tumor implantation. Nude mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. HepG2 tumor xenografts were established by subcutaneous injection of 5 × 10⁶ HepG2 cells/site in each mouse (pcDNAs5-TO-PV-NLS-DsRed, PV-NES-DsRed or DsRed). To express PV constructs, 100 μg/ml of doxycycline was added to the drinking water. Tumor measurement began when the tumor dimension reached 5 × 4 mm³ (~15 days after injection of the cells). HepG2 tumors were measured daily using a caliper, and the body weights of the mice were monitored as well. Tumor volume V was estimated by the formula V = (length) × (width)²/2 (20, 21).

**Ca²⁺ Measurements**—Nuclear and cytosolic Ca²⁺ were monitored in SKHepl cells using time lapse confocal microscopy (8, 12). SKHepl cells were cultured on glass coverslips, incubated for 30 min in the presence of 6 μM Fluo-4/AM to monitor nuclear or cytosolic Ca²⁺, and then transferred to a perfusion chamber on the stage of a Zeiss LSM 510 confocal microscope (Thornwood, NY). The cells were maintained in a HEPES-buffered solution during experiments and observed using a 63×, 1.4 numerical aperture objective lens. The 488-nm line of a krypton/argon laser was used to excite the dye, and emission signals between 505 and 550 nm were collected. The cells were stimulated with 100 nM vasopressin (Sigma), and the images were acquired at a rate of 2–10 frames/s. Neither autofluorescence nor background signals were detectable at the machine settings used.

**Statistical Analysis**—Changes in proliferation were determined by one-way analysis of variance. Significance of changes in treatment groups relative to controls were determined by t test. Significance of changes in the size of individual tumors over time was determined by two-way analysis of variance. The data are represented as the means ± S.E.

**RESULTS**

**Selective Buffering of Nuclear or Cytosolic Ca²⁺**—The relative role of nuclear and cytosolic Ca²⁺ signals in specific cell functions can be determined by selectively attenuating Ca²⁺ increases in either the nucleus or cytoplasm. The first studies in this area used microinjection of dextran-linked Ca²⁺ buffers into either the nucleus or cytoplasm (9, 22), but a more efficient approach was subsequently developed using targeted expression of Ca²⁺ buffering proteins such as PV or calretinin in these compartments (10, 23). This approach used constructs encoding PV targeted to the nucleus with a nuclear localization sequence (PV-NLS) or to the cytoplasm with a nuclear export sequence (PV-NES). To examine the effects of targeted Ca²⁺ buffering in entire cell populations, we delivered these constructs to SKHepl cells using an adenoviral vector to achieve high transfection efficiencies. The PV constructs were fused to DsRed to monitor expression and subcellular localization, which demonstrated the efficacy of the adenoviral vector (Fig. 1A). Consistent with previously published data (10, 23) time lapse confocal imaging confirmed that expression of PV-NLS and PV-NES was able to locally attenuate by >50% nuclear and cytosolic Ca²⁺ signals, respectively, in response to stimulation with vasopressin (Fig. 1B).

**Nuclear Ca²⁺ Controls Cell Proliferation**—To determine the involvement of nuclear and cytosolic Ca²⁺ in cell proliferation, SKHepl cells were synchronized in G₀ and then infected with the targeted PV constructs. The cells were stimulated to re-enter the cell cycle by the addition of serum. BrdUrd uptake was reduced by 63 ± 17% in cells expressing PV-NLS, compared with infected and uninfected cells (p < 0.001), whereas...
no reduction in BrdUrd uptake was seen in cells expressing PV-NES or in adenoviral infection controls (Fig. 2A). BrdUrd uptake was reduced to an intermediate degree (28% ± 17%; p = 0.001) in cells infected with a mutant PV-NLS in which one of the two Ca2+-binding sites was disrupted (Fig. 2A). This PV-NLS-CD mutant is known to have an intermediate effect on inhibition of nuclear Ca2+ signals, relative to PV-NLS (10). Cell proliferation in response to serum stimulation in PV-NLS- and PV-NES-DsRed controls confirm that proliferation is reduced by expression of PV-NLS but not PV-NES.

FIGURE 2. Cell growth depends on nuclear rather than cytosolic Ca2+. A, BrdUrd incorporation is decreased in SKHep1 cells expressing PV in the nucleus but not the cytoplasm, *, p < 0.001. BrdUrd incorporation decreases by an intermediate amount in cells expressing the CD mutant of PV in the nucleus, in which one of the two Ca2+-binding sites is disrupted. BrdUrd uptake is not decreased in DsRed adenoviral infection controls. The results are representative of what was observed in three separate experiments. B, cell growth curves of SKHep1 cells 24, 48, and 72 h after infection with targeted PV constructs confirm that proliferation is reduced by expression of PV-NLS but not PV-NES.
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PV-NES-expressing cells were also measured directly by cell counting. Like BrdUrd uptake, cell proliferation was reduced by buffering nuclear but not cytoplasmic Ca\(^{2+}\) (Fig. 2B). To determine whether the apparent decrease in cell proliferation was due in part to an increase in cell death, apoptosis was monitored using caspase-3 activity (24) (Fig. 3A) as well as Hoechst labeling of the nucleus (19) (Fig. 3B). Together, these findings demonstrate that cell proliferation is dependent upon increases in Ca\(^{2+}\) that occur in the nucleus but not the cytoplasm.

Buffering Nuclear Ca\(^{2+}\) Alters Cell Cycle Kinetics—We used flow cytometry analysis to understand which phases of the cell cycle are regulated by nuclear Ca\(^{2+}\). The cells were synchronized in Go, then released into the cell cycle by addition of serum, infected with adenoviral PV constructs, and then studied 48 h later. In populations expressing PV-NLS, there was a significant (\(p < 0.001\)) reduction in the fraction of cells in G\(_1\) phase (58 \(\pm\) 2%), but an increase in the fraction of cells in the S (14 \(\pm\) 1%) and G\(_2\)/M (27 \(\pm\) 1%) phases relative to uninfected controls (82 \(\pm\) 0.3%, 5 \(\pm\) 0.1%, and 12 \(\pm\) 0.4% in the G\(_1\), S, and G\(_2\)/M phases, respectively). No such change was observed in cells expressing PV-NES or in infected controls (Fig. 4A). These findings suggest that buffering nuclear Ca\(^{2+}\) specifically blocks either the G\(_2\)/M transition or mitosis (25, 26).

Progression through the cell cycle is regulated at various checkpoints by activation of specific complexes of cyclins and cyclin-dependent kinases (27, 28). These checkpoint proteins ensure that events comprising each phase of the cell cycle have completed before the next phase is initiated (27). To determine whether expression of PV-NLS was specifically affecting the G\(_2\)/M transition, we examined expression of the G\(_2\)/M checkpoint proteins cyclin B1 and cdk1. The expression of total cyclin B1 and cdk1 were unchanged, but phosphorylation of cdk1 was decreased (Fig. 4B). Because dephosphorylation activates rather than inhibits cdk1, these results suggest that the inhibitory effects of PV-NLS occur after rather than at the G\(_2\)/M transition. To confirm that PV-NLS expression does not inhibit progression through earlier phases of the cell cycle, we investigated expression of the G\(_1\)/S and S/G\(_2\) checkpoint proteins cyclins D1/cdk4 and cyclin E/cdk2, respectively. Buffering nuclear Ca\(^{2+}\) did not alter expression of any of these checkpoint proteins (Fig. 4, A and C). Similarly, buffering nuclear Ca\(^{2+}\) was associated with no change in the expression of the cyclin D1/cdk4 inhibitor p15 and decreased expression of the cyclin E/cdk2 inhibitor p27 and the cyclin B1/cdk1 inhibitor p21 (Fig. 4C). Interestingly, both expression and phosphorylation of the cdk1 phosphatase cdc25c was decreased in cells expressing PV-NLS (Fig. 4C), even though the net effect in these cells was dephosphorylation of cdk1 (Fig. 4B), which perhaps reflects that multiple factors contribute to the regulation of cdk1 activity (29, 30).

As an additional way to examine the effects of nuclear Ca\(^{2+}\) on cell proliferation, the effect of PV-NLS on the growth-related kinases ERK1/2, ERK5, and AKT and the transcription factor CREB were examined. Buffering cytoplasmic rather than nuclear Ca\(^{2+}\) decreased the level of p-ERK1/2, and PV-NLS had no effect on total expression of ERK1/2, ERK5, or AKT (Fig. 4D). However, expression of PV-NLS decreased p-CREB (Fig. 4D), consistent with previous reports that this transcription factor is activated by nuclear rather than cytosolic Ca\(^{2+}\) (9). Together, these observations suggest that nuclear Ca\(^{2+}\) acts during rather than before mitosis to regulate cell proliferation.

Nuclear Ca\(^{2+}\) Controls Progression through Mitosis—To investigate the effects of nuclear and cytoplasmic Ca\(^{2+}\) on mitosis, cells were labeled with the mitotic marker phospho-histone-3 and the centrosome marker \(\gamma\)-tubulin 48 h after infection with the various PV constructs. Labeled cells were examined by confocal microscopy (Fig. 5A) to determine the fraction of cells in mitosis. The mitotic index was doubled in cells in which nuclear Ca\(^{2+}\) was buffered (\(p < 0.001\)) and was no different from controls in cells in which cytoplasmic Ca\(^{2+}\) was buffered (Fig. 5B). Examination of the phospho-histone-3 pattern in cells expressing PV-NLS furthermore suggested that
these cells were in early prophase, and γ-tubulin staining similarly demonstrated that only a single centrosome was present in these cells (Fig. 5C). Analysis of the phospho-histone-3 staining pattern in all mitotic cells within each treatment group confirmed that expression of PV-NLS induced a block in early prophase (Fig. 5D). Specifically, 27 ± 2% of cells expressing PV-NLS were in prophase, and none were in metaphase or anaphase, whereas in control cells 12 ± 1% were in prophase, 2 ± 0.3% were in metaphase, and 3 ± 0.3% were in anaphase ($p < 0.001$). These results demonstrate that nuclear Ca$^{2+}$ is necessary specifically for centrosome separation and progression through early prophase.

Nuclear Ca$^{2+}$ Is Needed for Tumor Growth in Vivo—To understand the potential significance of these findings in vivo, we examined the effects of nuclear and cytoplasmic Ca$^{2+}$ signals on the growth of tumors implanted into nude mice. The HepG2 tumor cell line was stably transfected with PV-NLS-DsRed, PV-NES-DsRed, or DsRed alone, each under the control of a doxycycline-sensitive promoter (Fig. 6A). BrdUrd uptake in these cells was reduced by over 60% ($p < 0.001$) by expression of PV in the nucleus but not the cytosol (Fig. 6B), similar to what was observed in SkHep1 cells (Fig. 2A). These HepG2 cells were implanted into the subcutaneous space, and then tumor growth was measured over a two-week period with or without administration of doxycycline in the drinking water (20, 21). Immunoblot analysis of tumors removed 2 weeks after implantation confirmed expression of the targeted PV constructs in doxycycline-treated animals and not in untreated controls (Fig. 6C). Tumor growth was significantly impaired by expression of PV-NLS-DsRed ($p < 0.001$) but not by expression of PV-NES-DsRed or DsRed alone (Fig. 7). Direct examination of resected tumors (not shown) suggested an even greater inhibition of tumor growth by PV-NLS-DsRed than was appreciated by in vivo measurements, perhaps because caliper measurements include overlying skin and associated tissues as well. These findings confirm the role of nuclear rather than cytoplasmic Ca$^{2+}$ signals in the regulation of cell growth.

**DISCUSSION**

It is well appreciated that increases in Ca$^{2+}$ occur in both the cytoplasm and the nucleus. Initial evidence suggested that nucleoplasmic Ca$^{2+}$ passively follows changes in cytoplasmic Ca$^{2+}$ (31, 32). However, subsequent work established that the nucleus contains distinct Ca$^{2+}$ stores (7), including a nucleoplasmic reticulum (12), plus phosphatidylinositol (4,5)biphosphate (33), phospholipase C (34), and InsP3 receptors (12, 35). Together, these factors provide the machinery necessary to permit nucleoplasmic Ca$^{2+}$ to be regulated independently from cytoplasmic Ca$^{2+}$ (8). The significance of this machinery within the nucleus has long been a question. Both growth factors (12) and integrins (36) can preferentially increase nucleoplasmic Ca$^{2+}$, consistent with the idea that nucleoplasmic Ca$^{2+}$ would be more important than cytoplasmic Ca$^{2+}$ to regulate cell proliferation. Increases in

**FIGURE 4.** Buffering nuclear Ca$^{2+}$ suggests a block in late G2 or M phase. A, fluorescence activated cell sorting analysis of DNA content performed 48 h after infection with the indicated PV constructs or controls. In cells expressing PV-NLS-DsRed, there is an 8 and 16% increase in the fraction of cells in S and G2 phase, respectively, and a 24% decrease in cells in G1, suggesting a block in late G2 or M phase. Cell cycle profiles are confirmed that expression of PV-NLS induced a block in early prophase.

**Nuclear Ca$^{2+}$ and Cell Growth**
Nuclear Ca\textsuperscript{2+} and Cell Growth

A

| Stage         | γ-tubulin | histone-3 | hoechst | merged |
|---------------|-----------|-----------|---------|--------|
| early prophase|           |           |         |        |
| late prophase |           |           |         |        |
| prometaphase  |           |           |         |        |
| metaphase     |           |           |         |        |
| anaphase      |           |           |         |        |

B

![Bar chart showing mitotic index (% of cells) for different conditions](image)

C

![Images showing γ-tubulin and histone-3](image)

D

![Bar chart showing mitotic index (% of cells) for different conditions](image)
InsP3 (16, 37), plus temporally related increases in Ca\(^{2+}\)/H\(_{11545}\) in the perinuclear region (26, 38, 39), have been associated with breakdown of the nuclear envelope and entry into mitosis. In addition, nuclear Ca\(^{2+}\)/H\(_{11545}\) activates transcription factors such as CREB (9, 11), Elk-1 (10), and DREAM (40), but cytosolic Ca\(^{2+}\)/H\(_{11545}\) activates other transcription factors such as SRE (9), plus phosphatases such as calcineurin that are important for transcription (41). Therefore, the relative importance of nuclear and cytosolic Ca\(^{2+}\)/H\(_{11545}\) to cell cycle progression is highly dependent on the context of the cell and the stage of the cell cycle.

**FIGURE 5.** Nuclear Ca\(^{2+}\)/H\(_{11545}\) regulates progression through early prophase. A, representative images of SKHep1 cells in early and late prophase, metaphase, and anaphase. These confocal immunofluorescence images were obtained after staining with anti-phospho-histone-3 (green) and anti-\(\gamma\)-tubulin (pink) to label each phase of mitosis, plus Hoechst (blue) to label the nucleus. B, the mitotic index is increased in cells in which nuclear Ca\(^{2+}\)/H\(_{11545}\) is buffered. *, \(p < 0.001\). Mitotic SKHep1 cells were identified by histone-3 labeling, measured 48 h after infection with the indicated PV constructs. A total of 200 cells were examined in each experiment, and each experiment was conducted six times. C, representative image of a mitotic SKHep1 cell in which nuclear Ca\(^{2+}\)/H\(_{11545}\) is buffered with PV-NLS-DsRed. Serial confocal sections (one of which is shown here) reveal a single centrosome by \(\gamma\)-tubulin staining, and mitotic figures illustrate of early prophase by histone-3 staining. D, mitotic index, subdivided to demonstrate the fraction of cells in each phase of mitosis, demonstrates that cells in which nuclear Ca\(^{2+}\)/H\(_{11545}\) is buffered do not progress beyond prophase. *, \(p < 0.001\). A total of 200 cells were examined in each experiment, and the experiments were performed in quadruplicate.

**FIGURE 6.** Regulated expression of targeted PV constructs in a tumor cell line. A, immunoblot analysis of the engineered HepG2 stable cell lines after treatment using the indicated concentrations (62.5–1000 ng/ml) of doxycycline to induce expression of PV-NLS or PV-NES. B, BrdUrd incorporation is decreased in HepG2 cells expressing PV in the nucleus but not the cytoplasm. *, \(p < 0.001\). BrdUrd uptake is not decreased in controls that inducibly express DsRed. The results are representative of what was observed in three separate experiments. C, the phenotype of the engineered HepG2 cells is retained after implantation into nude mice. Immunoblot analysis of tumors removed 2 weeks after implantation confirms parvalbumin expression is preserved in PV-NLS and PV-NES but not DsRed tumors and only in tumors from animals treated with doxycycline. The slight difference in molecular weight between PV-NLS and PV-NES tumors reflects differences in weight caused by the targeting sequences.

**FIGURE 7.** Nuclear Ca\(^{2+}\)/H\(_{11545}\) regulates cell growth in vivo. Nuclear but not cytoplasmic Ca\(^{2+}\)/H\(_{11545}\) buffers retard tumor growth. HepG2 tumor cells expressing PV-NES-DsRed, PV-NLS-DsRed, or DsRed under the control of a doxycycline-sensitive promoter were implanted subcutaneously into nude mice. Serial measurements of tumor volume in the presence or absence of doxycycline (100 \(\mu\)g/ml, added to the drinking water) were obtained, starting when tumor volume reached 5 \(\times\) 4 mm\(^3\). The data represent the means \(\pm\) S.E. of tumor volume over time (\(n = 5–15\) mice in each group), normalized with respect to the initial volume. *, \(p < 0.001\).
cytoplasmic Ca\(^{2+}\) for regulating cell growth has been hypothesized but not proven from these previous studies. The current work directly demonstrates that nuclear but not cytoplasmic Ca\(^{2+}\) regulates cell proliferation and that this occurs in multiple cell lines and in vivo as well as in vitro.

The current work raises several new issues of fundamental importance. First, our findings suggest a paradigm in which InsP\(_3\) formation in the cytoplasm regulates Ca\(^{2+}\)-mediated events that are cell type-specific, such as secretion or contraction, whereas InsP\(_3\) formation in the nucleus instead regulates the cell cycle. This in turn suggests that regulatory pathways would exist to bypass cytoplasmic InsP\(_3\) formation to stimulate nuclear \(\text{Ca}^{2+}\) work directly demonstrates that nuclear but not cytoplasmic \(\text{Ca}^{2+}\) regulates cell proliferation and that this occurs in multiple cell lines and in vivo as well as in vitro. Second, our findings demonstrate that \(\text{Ca}^{2+}\) work directly demonstrates that nuclear but not cytoplasmic \(\text{Ca}^{2+}\) regulates cell proliferation and that this occurs in multiple cell lines and in vivo as well as in vitro. Therefore, it will be important to understand whether and how InsP\(_3\) formation within the nucleus occurs. Second, our findings demonstrate that cytoplasmic \(\text{Ca}^{2+}\) regulates cell cycle progression even in tumor cells. One benefit of these findings is that this effect could be used to directly inhibit the growth of tumors, as was demonstrated here. However, buffering nuclear \(\text{Ca}^{2+}\) induces a mitotic block but does not induce apoptosis, so this strategy would not be expected to decrease the overall tumor burden. Therefore an alternative strategy might be to buffer nuclear \(\text{Ca}^{2+}\) in tumor cells over a specific time interval to induce and then release the mitotic block in a synchronized fashion. This in turn could synchronize the tumor cells within the cell cycle, rendering them more uniformly susceptible to existing chemotherapeutic agents that destroy only cells at specific phases of the cell cycle (42). As mechanisms for the regulation of nuclear \(\text{Ca}^{2+}\) are identified, this may lead to development of new strategies to regulate abnormal growth of cells.

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