The Journal of Neuroscience, January 1, 2000, *20*(1):315–325

Regulation of Calcineurin by Growth Cone Calcium Waves Controls Neurite Extension

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Growth cones generate spontaneous transient elevations of intracellular Ca^{2+} that regulate the rate of neurite outgrowth. Here we report that these Ca^{2+} waves inhibit neurite extension via the Ca^{2+}-dependent phosphatase calcineurin (CN) in *Xenopus* spinal neurons. Pharmacological blockers of CN (cyclosporin A and deltamethrin) and peptide inhibitors of CN [the *Xenopus* CN (xCN) autoinhibitory domain and African swine fever virus protein A238L] block the Ca^{2+}-dependent reduction of neurite outgrowth in cultured neurons. Time-lapse microscopy of growing neurites demonstrates directly that the reduction in the rate of outgrowth by Ca^{2+} transients is blocked by cyclosporin A. In contrast, expression of a constitutively active form of xCN in the absence of waves results in shorter neurite lengths similar to those seen in the presence of waves. The developmental expression pattern of xCN transcripts *in vivo* coincides temporally with axonal pathfinding by spinal neurons, supporting a role of CN in regulating Ca^{2+}-dependent neurite extension in the spinal cord. Ca^{2+} wave frequency and Ca^{2+}-dependent expression of GABA are not affected by inhibition or activation of CN. However, phosphorylation of the cytoskeletal element GAP-43, which promotes actin polymerization, is reduced by Ca^{2+} waves and enhanced by suppression of CN activity. CN ultimately acts on the growth cone actin cytoskeleton, because disrupting actin microfilaments with cytochalasin D or stabilizing them with jasplakinolide negates the effects of suppressing or activating CN. Destabilization or stabilization of microtubules with colcemide or taxol results in Ca^{2+}-independent inhibition of neurite outgrowth. The results identify components of the cascade by which Ca^{2+} waves act to regulate neurite extension.

Key words: axonal outgrowth; calcium transients; actin; microtubules; GAP-43; PP1; PP2A; PP2B

Ca^{2+} transients encode information across a range of frequencies (Berridge and Rapp, 1979; Tsien and Tsien, 1990; Meyer and Stryer, 1991; Spitzer and Sejnowski, 1997) and direct distinct aspects of differentiation in spinal neurons (Gu et al., 1994; Gu and Spitzer, 1995; Gomez and Spitzer, 1999). Growth cone Ca^{2+} waves regulate the rate of axon extension, which is inversely proportional to their frequency. The effects of Ca^{2+} transients on growth cone motility have been extensively studied (Haydon et al., 1984; Cohan and Kater, 1986; Mattson et al., 1988; Fields et al., 1990; Gomez et al., 1995), but the basis of decoding Ca^{2+} transients on this slow time scale, over periods of hours rather than seconds, has remained elusive. Ca^{2+}–calmodulin kinase II (CamKII) has been implicated as a frequency decoder of Ca^{2+} transients (Deisseroth et al., 1995, 1998; De Koninck and Schulman, 1998). However, neurite outgrowth of spinal neurons is not affected by inhibitors of CamKII (Zheng et al., 1994) or of Ca^{2+}-dependent protein kinase (PKC) (Gu and Spitzer, 1995), implying that another Ca^{2+}-dependent enzyme is involved.

Calcineurin (CN) is a Ca^{2+}- and calmodulin-dependent protein phosphatase that has been reported to either stimulate or inhibit neurite outgrowth depending on cell type (Ferreira et al., 1993; Lyons et al., 1994; Chang et al., 1995). Local inactivation of CN causes temporary filopodial retraction in the growth cones of cultured neurons (Chang et al., 1995). Moreover, growth cones severed from their axons retain the ability to modulate filopodia in response to Ca^{2+} elevations (Rehder and Cheng, 1998), suggesting that CN acts on the local cytoskeleton. It is unlikely that CN affects neurite extension over the short term by regulating transcription, because neuronal morphology appears normal when inhibitors of RNA synthesis are applied (Ribera and Spitzer, 1989).

We investigated the role of CN in Ca^{2+}-dependent regulation of axonal outgrowth in cultured *Xenopus* spinal neurons. Neurite extension increases when CN is suppressed pharmacologically in the presence of extracellular Ca^{2+} but not in its absence, implicating it in the transduction cascade. Accordingly we cloned *Xenopus* calcineurin (xCN) to be able to manipulate its activity. Expression of the autoinhibitory domain of xCN in the presence of Ca^{2+} waves increases neurite extension, whereas expression of a constitutively active form of xCN in the absence of Ca^{2+} waves retards neurite growth. The frequency of Ca^{2+} waves is not affected by inhibiting or activating CN, positioning the action of CN downstream of waves. Antibodies specific to phosphorylated GAP-43 reveal that Ca^{2+} waves act via CN to dephosphorylate GAP-43, consistent with the destabilization of actin filaments that slows neurite extension. The effects of suppressing or activating CN are blocked by destabilizing or stabilizing actin microfilaments but not microtubules, suggesting that CN acts on the growth cone actin cytoskeleton. The pattern of xCN expression in

Received June 29, 1999; revised Oct. 14, 1999; accepted Oct. 15, 1999.

This work was supported by the National Institute of Neurological Disorders and Stroke Grant NS15918 and the National Institutes of Health Grant 5T32GN08107. We thank Steve Watt, Clay McDaniels, and Bill Conroy for valuable technical assistance, Stephen O'Keefe for providing the murine calcineurin B clone, Linda Dixon for providing the viral A238L clone, Karina Meiri for the rat GAP-43 construct and anti-GAP-43 antibodies, and Tim Gomez and Ray Smith for their comments on this manuscript.

The GenBank accession number for the *Xenopus* calcineurin A gene is AF019569.

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the developing spinal cord is temporally and spatially appropriate to be involved in the epigenetic mechanisms that regulate neurite outgrowth. Thus activation of xCN by spontaneous Ca^{2+} transients may control axon extension *in vivo*.

MATERIALS AND METHODS

Cell culture and expression of cRNA. Adult *Xenopus laevis* females were primed with human chorionic gonadotropin (United States Biochemicals, Cleveland, OH), oocytes were fertilized *in vitro,* and embryos were allowed to develop to the neural plate stage (stage 15) (Nieuwkoop and Faber, 1967). For most experiments, including those involving application of pharmacological agents or expression of constructs, the presumptive spinal cord was isolated, disaggregated in a solution lacking divalent cations, and plated in a culture medium containing either 10 or 0 mM Ca^{2+} (116 mM NaCl, 0.67 mM KCl, 1.31 mM $MgSO_4$, either 10 mM $CaCl_2$ or 0 mM $CaCl_2$ with 1 mM EGTA, and 4.6 mM Tris; pH adjusted to 7.8 with HCl) to promote or inhibit generation of spontaneous Ca^{2+} waves. For experiments involving expression of RNA constructs, fertilized oocytes were treated with 2% cysteine and 10% Holtfreter's solution, pH 8, to remove the vitelline membrane. Injections of 10–20 ng of cRNA into one cell of two-cell stage embryos were performed in a solution of 6% Ficoll in 10% MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 0.1 mM EDTA, and 2 mM $CaCl_2$; pH adjusted to 7.8 with NaOH). A fluorescent marker (FITC-, rhodamine-, or fura-2-dextran; Molecular Probes, Eugene, OR) was coinjected with the cRNA to allow rapid identification of cells expressing constructs; nonfluorescent cells provided an internal control. Neurons were allowed to develop, and assays were performed at 18 to 24 hr after plating. The effects of pharmacological inhibitors on neurite length were scored by measuring the length of the longest neurite for the first 10 neurons encountered in experimental and in control cultures. The effects of a CN autoinhibitory peptide, viral A238L protein, and constitutively active (CA)-xCN expression on neurite length were determined for the first 10 fluorescent and 10 nonfluorescent neurons in the same culture. For experiments involving measurements of neurite growth rate in response to Ca^{2+} transients generated by photorelease of caged Ca^{2+}, neurons were grown on a laminin substrate to promote more rapid extension. Laminin (25 μg/ml; Sigma, St. Louis, MO) was coated onto acid-washed glass coverslips for 6 hr at room temperature (20°C) and washed with sterile PBS, pH 7.4. Neural explants (stage 21) were dissected in the presence of collagenase B (0.15 mg/ml; Boehringer Mannheim, Indianapolis, IN), washed, and plated in culture medium containing 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6. Data are presented as mean ± SEM, using a double-blind assignment; $n \geq 100$ neurons from three or more culture dishes unless otherwise indicated. Values are considered significantly different when $p < 0.01$ unless denoted otherwise.

Pharmacological studies. Cyclosporin A (CsA), deltamethrin (DM), okadaic acid (OA), rapamycin (Rap), and tautomycin (Taut) were obtained from Calbiochem (San Diego, CA). Colcemide, cytochlasin D (Cyto D), jasplakinolide (Jasp), and taxol were from Molecular Probes. All drugs were stored at −20°C in the dark. CsA and DM were made fresh for each experiment, dissolved in a cocktail of 50% ethanol and 50% DMSO, and then added to warmed (37°C) culture medium and allowed to cool to room temperature. Colcemide, Cyto D, OA, Rap, Taut, and taxol were dissolved in DMSO, aliquoted for single use, and stored at −20°C in the dark. Jasp was dissolved in methanol. CsA, DM, OA, Rap, and Taut were added 4 hr after plating; colcemide, Cyto D, Jasp, and taxol were added 7 hr after plating to allow neurite initiation. Final ethanol, methanol, and DMSO concentrations were <0.001%. Dose–response curves were determined for each agent to identify the minimum effective concentration.

Isolation of Xenopus *calcineurin A.* A partial clone of *Xenopus* CN A, 1068 bp in length, was isolated from a 1 month *Xenopus* tadpole brain cDNA library. Because the partial clone lacked the 5′ portion of the CN gene, it was isolated by 5′ rapid amplification of cDNA ends (RACE; Life Technologies, Gaithersburg, MD). A single gene-specific primer (gtcatggtaaccagagacttc) was used to generate a 1 kb fragment including 300 bp of overlap with the original clone from stage 30 spinal cord mRNA. A second gene-specific nested primer (ggcctacaagtactgtaataag) was used in conjunction with a 5′-tailed primer for exponential amplification of the 5′ fragment. The 5′ fragment was isolated using the TA cloning kit (Invitrogen, San Diego, CA) and ligated to the partial clone at a unique Bst 1107 I site.

Inhibitory and constitutively active constructs. A PCR-based approach was used to generate constructs encoding two peptide inhibitors specific for CN and a constitutively active form of the enzyme. The large autoinhibitory domain of CN was isolated using primers (forward, aggcctaaaggtcttacacccaccg; reverse, tctagatcactgaatattgctgccg) based on the work of Sagoo et al. (1996), generating a 291 bp product. The African swine fever virus protein A238L sequence (Miskin et al., 1998) (construct kindly provided by Dr. Linda Dixon) was isolated in a similar manner (forward, aggcctatggaacacatgtttcc; reverse, ctcgagcggccgccagtgtgatgg), generating a 704 bp product. A similar approach was used to generate a CA construct that is Ca^{2+} and calmodulin independent (CA-xCN), with primer design based on previously published work (O'Keefe et al., 1992) to isolate the 1.2 kb catalytic region of CN (forward, gaattcaatgtccgagcacaagg; reverse, tctagatcactagtttctgataacttcc). All PCR products were subcloned into pCR2.1 (Invitrogen) and later subcloned into CS2+MYC with five consecutive myc tags (designed by Dr. David Turner). Each insert was sequenced to verify the integrity of the PCR product. mRNA was synthesized using the SP6 mMessage machine mRNA transcription kit (Ambion, Austin, TX) and injected into one cell of a two-cell stage *Xenopus* embryo along with a fluorescent marker. CA-xCN was coinjected with murine CN B cRNA (construct generously provided by Dr. Stephen O'Keefe) to enhance stability of the enzyme.

In situ *hybridization. In situ* hybridization of *Xenopus* embryos was performed as described previously (Harland, 1991; Ferreiro et al., 1992), with modifications (Burger and Ribera, 1996). Antisense and sense RNA probes were constructed containing 1 kb of the 3′ untranslated region and 400 bp of the coding region. To facilitate visualization after staining, we used albino *Xenopus* embryos obtained from mated-pair fertilizations.

Ca^{2+} imaging. Relative levels of intracellular Ca^{2+} were measured using Fluo 3 AM or 4 AM (Molecular Probes). Cells were loaded with 5 μM Ca^{2+} indicator or with 2.5 μM Ca^{2+} indicator and 2.5 μM NP-EGTA AM (caged Ca^{2+}) (in 0.02% pluronic acid and 0.2% DMSO) for 60 min and then perfused with culture medium. Individual cultures were imaged for not >75 min. Measurements of Ca^{2+} transients produced in response to photorelease of caged Ca^{2+} from NP-EGTA AM and determinations of consequent rates of neurite extension were performed with a Bio-Rad MRC600 confocal microscope (Hercules, CA). Images were acquired at 15 sec intervals at 10–18 hr in culture using a 100× objective. Neurite outgrowth was measured over 24 min intervals, after photorelease of caged Ca^{2+} at 8 and 16 min; data were tabulated from neurons in which extension recovered after photorelease. Fluorescence pixel intensities were analyzed with NIH Image (W. Rasband, National Institutes of Health, Bethesda, MD), changes were normalized to baseline, and waves were scored as events >150% of their baseline fluorescence (F/F_0) that were distinguished from spikes by their kinetics (Gu et al., 1994). Time-lapse measurements of spontaneous Ca^{2+} transients and identification of fluorescent cells for neurite length assays were accomplished with a TTE CCD camera mounted on a Zeiss IM-35 Photoscope. Images were captured at 10 sec intervals at 12–24 hr in culture using 20× or 40× water immersion objectives. Fluorescence pixel intensities were analyzed with the Metamorph program (Universal Imaging Corporation, West Chester, PA), and waves were scored as described above.

Immunocytochemistry. Analysis of myc immunoreactivity followed previously described protocols, with modifications (Spitzer et al., 1993; Gu and Spitzer, 1995). Cells were fixed with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde, washed in PBS, and incubated with an anti-myc mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 overnight at 4°C. A secondary biotinylated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) was used at 1:500 for 2 hr at 20°C. Avidin-conjugated horseradish peroxidase and diaminobenzidine or NovaRed substrate (Vector Laboratories, Burlingame, CA) were used to identify myc-positive neurons. GABA immunoreactivity was detected in an identical manner to myc, using a rabbit anti-GABA polyclonal primary antibody (Incstar, Stillwater, MN) at 1:5000 and a tetramethylrhodamine isothiocyanate-biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) at 1:500.

Immunoblot analysis. Neural plates from embryos injected with rat GAP-43 cRNA (construct generously provided by Dr. Karina Meiri) were cultured as described above and harvested 18–24 hr after plating; 15 or more cultures were pooled to allow detection of GAP-43. Protein was solubilized in SDS sample buffer containing 5% β-mercaptoethanol, subjected to SDS-PAGE on 8% polyacrylamide gels, and electroblotted on nitrocellulose. Blots were blocked with 3% (w/v) Carnation nonfat dry milk in PBS containing 0.1% Tween 20, incubated overnight at 4°C

with anti-GAP-43 mAb 2G12 (courtesy of Dr. Karina Meiri) at 1:10 in the same solution, washed in PBS containing 0.1% Tween 20, and then incubated for 1 hr at 20°C with horseradish peroxidase coupled to goat anti-mouse IgG (Jackson ImmunoResearch) to detect bound mAb. Signals were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL), digitized with a Color One scanner (Apple Computer, Cupertino, CA), and band intensities were quantitated using NIH Image software (W. Rasband, National Institutes of Health). Molecular mass markers for blots included phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa) (low range; Bio-Rad).

RESULTS

Pharmacological inhibition of calcineurin enhances neurite outgrowth

Growth cone Ca^{2+} waves are abolished and neurites are significantly longer when neurons are grown in the absence of external Ca^{2+} rather than in its presence, consistent with previous observations (Bixby and Spitzer, 1984; Gu et al., 1994; Gu and Spitzer, 1995). We investigated the regulation of neurite extension by the Ca^{2+}-dependent protein phosphatase calcineurin (PP2B) by applying cyclosporin A (CsA; 10 nM) and dellamethria (DM; 1 nM) to cultures in the absence or presence of Ca^{2+} to eliminate or enable spontaneous production of waves. CsA binds to cyclophilin A and inhibits CN indirectly (Liu et al., 1991), whereas DM apparently binds directly to CN (Enan and Matsumura, 1992). Inhibition of CN has no effect on neurite length in the absence of Ca^{2+} waves. However, in the presence of Ca^{2+} waves, inhibition of CN generates longer neurite lengths, resembling those observed in 0 Ca^{2+} (Fig. 1*A*; $p < 0.01$). Higher concentrations of CsA (1 μM) and DM (100 nM) inhibit neurite extension in a Ca^{2+}-independent manner, consistent with previous observations (Chang et al., 1995), presumably by interfering with other cellular functions. Lower concentrations have no effect on neurite outgrowth. Immunophilins implicated in axonal outgrowth (Lyons et al., 1994; Chang et al., 1995; Gold, 1997; Snyder at al., 1998) do not appear to regulate Ca^{2+}-dependent outgrowth because rapamycin (Rap), which binds immunophilins but does not inhibit CN, has no effect on neurite length (control, 92 ± 6 μm; 1 μM Rap, 100 ± 6 μm; $n > 30$). Thus CN seems to be a necessary component in the signal transduction cascade by which Ca^{2+} waves control the rate of neurite extension *in vitro*.

Because CN can affect the activity of other serine–threonine phosphatases, we determined whether they regulate neurite extension in response to Ca^{2+} waves by testing inhibitors of the PP2A and PP1 families of protein phosphatases. Okadaic acid (OA) is a potent inhibitor of PP2A members at low concentrations and PP1 members at higher concentrations (for review, see MacKintosh and MacKintosh, 1994). The dose–response relationship for OA reveals that PP2A members promote neurite growth in the absence of Ca^{2+} waves rather than inhibit it in a Ca^{2+}-dependent manner (Fig. 1*B*). At 300 pM, OA has no effect on neurite length, as expected, because this concentration is below the levels that inhibit phosphatase activity (Favre et al., 1997). Specific inhibition of PP2A family members (3 nM) results in neurite lengths that are reduced only in the absence of extracellular Ca^{2+}. Increasing the OA concentration to 30 nM, which inhibits PP1 as well as PP2A family members, leads to no significant further reduction in neurite lengths. These results suggest that the activity of PP2A but not PP1 phosphatases stimulates neurite growth in the absence of Ca^{2+} waves; however neither family acts downstream of Ca^{2+} waves to inhibit neurite outgrowth. To identify the contribution of PP1 phosphatases we used Taut, which specifically inhibits this family at low concentrations

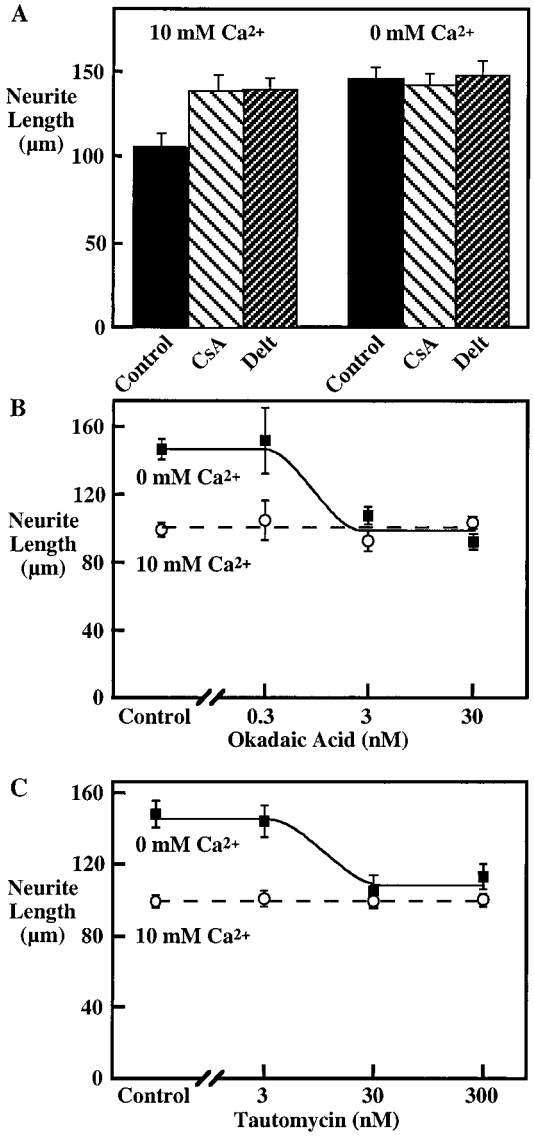

Figure 1. Pharmacological inhibition of CN but not other ser–thr phosphatases increases neurite lengths in the presence of Ca^{2+}. *A,* Neurite lengths are significantly increased by 10 nM CsA or 1 nM DM (*Delt*) in the presence of 10 mM Ca^{2+}. In the absence of extracellular Ca^{2+}, inhibition of CN (PP2B) by 10 nM CsA or 1 nM DM has no effect on neurite lengths. Data are mean ± SEM ($n \geq 100$). *B,* OA at 300 pM, a concentration that does not inhibit members of the PP1 or PP2A families of protein phosphatases, has no effect on neurite length ± Ca^{2+}. OA at 3 nM, which inhibits members of PP2A but not PP1, inhibits neurite lengths in the absence of Ca^{2+}, suggesting that this family promotes neurite extension in the absence of waves. OA at 30 nM, which inhibits members of both PP2A and PP1, causes no further inhibition of neurite outgrowth. *C,* Taut at 3 nM, a concentration that affects only PP1 and not PP2A phosphatases, has no effect on neurite extension ± Ca^{2+}. Taut at 30 and 300 nM, which inhibit members of both PP1 and PP2A, results in Ca^{2+}-independent inhibition of neurite lengths. Data for *B* and *C* are mean ± SEM ($n \geq 30$).

(MacKintosh and MacKintosh, 1994). At 3 nM, tautomycin (Taut) has no effect on neurite extension in the presence or absence of Ca^{2+} (Fig. 1*C*). Since CN inhibition can enhance PP1 activity by suppressing Inhibitor-1, a CN substrate that blocks PP1 when dephosphorylated, these data also make it unlikely that members of PP1 are responsible for the increase in neurite outgrowth in the absence of waves. In agreement with the effects of OA, higher

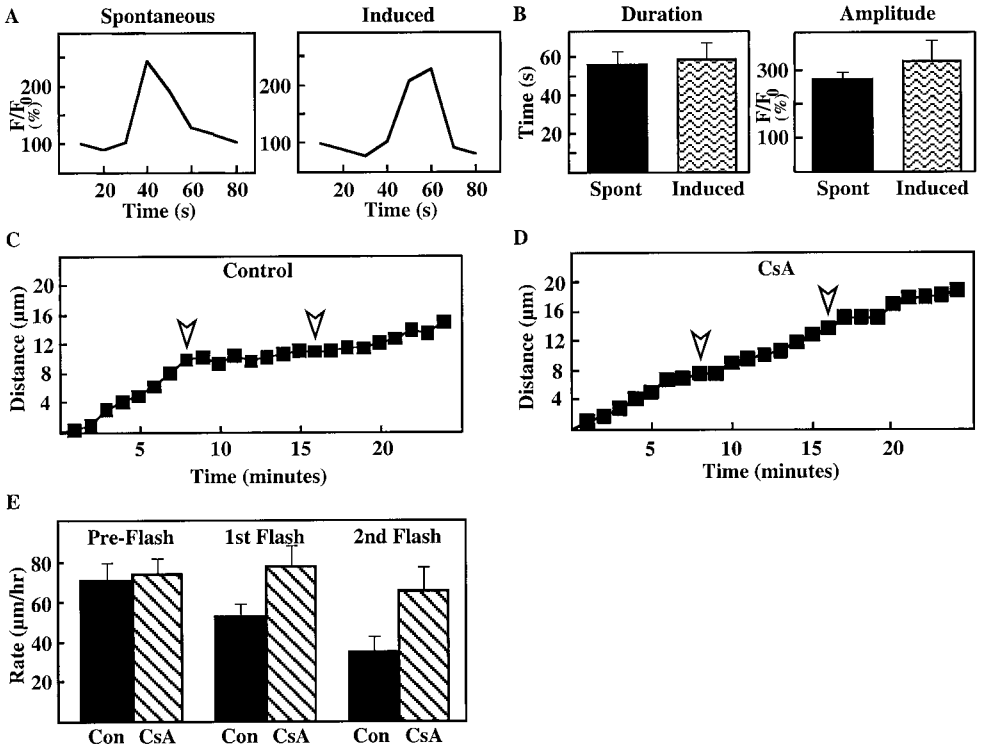

Figure 2. Inhibition of calcineurin suppresses the slowing of neurite outgrowth by Ca^{2+} transients generated by photorelease of caged Ca^{2+}. *A,* UV stimulation of a growth cone elicits a Ca^{2+} transient (*right*; laminin substrate) that mimics a spontaneous Ca^{2+} wave in a different growth cone (*left*; tissue culture plastic substrate). *B,* Induced Ca^{2+} transients mimic spontaneous growth cone Ca^{2+} waves in both duration and amplitude (mean ± SEM; $n = 5$). *C,* Growth cone Ca^{2+} transients evoked at 8 min intervals (*arrowheads*) inhibit the rate of outgrowth. *D,* Inhibition of calcineurin (CsA, 10 nM) negates the braking effect of Ca^{2+} transients (*arrowheads*) on neurite outgrowth. Neurons were imaged to establish a baseline rate of outgrowth, followed by photorelease of Ca^{2+} in the growth cone at 8 min intervals to mimic growth cone waves. *E,* Inhibition of neurite outgrowth by Ca^{2+} transients is suppressed in the presence of CsA (10 nM), suggesting that activation of calcineurin slows neurite outgrowth (mean ± SEM; $n \geq 15$; $p > 0.05$). *Con*, Control; *Spont*, spontaneous.

concentrations of tautomycin that inhibit members of the PP2A family (30 and 300 nM) suppress neurite lengths only in the absence of extracellular Ca^{2+}. Thus, neither PP2A nor PP1 phosphatase families seem to have a role in the Ca^{2+}-dependent inhibition of neurite extension in *Xenopus* spinal neurons.

Regulation of neurite extension by Ca^{2+} transients is suppressed by CsA

To test more directly whether CN slows neurite extension in response to Ca^{2+} transients, we simulated waves by focal photorelease of caged Ca^{2+} in the growth cone under conditions that permitted or suppressed CN activity. In preliminary experiments, photodynamic damage appeared to result from the stimulation of Ca^{2+} transients for the several hours necessary to detect changes in the relatively slow rate of neurite extension, when neurons were grown on uncoated culture dishes. Accordingly, we analyzed the more rapid neurite outgrowth achieved with neural tube explants grown on laminin. Laminin also suppresses spontaneous growth cone wave production, facilitating temporal control of Ca^{2+} elevations via photorelease. Cells were loaded with Fluo 3 AM and NP-EGTA AM (caged Ca^{2+}), and baseline rates of growth were established for each neuron. Subsequently Ca^{2+} was uncaged with brief UV flashes delivered to the growth cone (Gomez and Spitzer, 1999). Ca^{2+} transients generated in this way mimic spontaneous Ca^{2+} waves in their amplitude and duration (Fig. 2*A,B*). Moreover, imposition of Ca^{2+} transients at a frequency of 8/hr (Gu and Spitzer, 1992) slows the rate of neurite outgrowth. However in the presence of 10 nM CsA, the braking effect of Ca^{2+} waves on neurite outgrowth is significantly reduced (Fig. 2*C–E*). These data indicate that neurite outgrowth on a natural laminin substrate is regulated by Ca^{2+} transients, similar to growth on culture plastic and *in vivo*, and suggest that Ca^{2+} waves are linked to neurite extension via the activity of CN.

Cloning of *Xenopus* calcineurin

Because CN emerged as an important link between Ca^{2+} waves and reduced neurite outgrowth, we cloned the xCN A subunit. A 1.1 kb partial clone was isolated from a *Xenopus* brain cDNA library. The remaining 5′ portion of the gene was isolated using RACE and ligated with the original gene fragment to produce a full-length clone. xCN corresponds to the neuronal A isoform and has the highest identity with human, rat, and mouse calcineurin at the amino acid level (94%) based on BLASTx searches of GenBank (Altschul et al., 1997).

Peptide inhibition of calcineurin enhances neurite extension

To characterize further the effect of inhibition of endogenous CN, we used specific blockers to examine the role of CN in the regulation of neurite outgrowth by Ca^{2+} waves. Because xCN contains an autoinhibitory domain that inhibits enzymatic activity, we used a PCR-based approach to isolate various sized domains encoding the inhibitory region. PCR products were subcloned into an expression vector encoding five consecutive myc tags at the 5′ end of the sequence. cRNA was synthesized and coinjected into one cell of a two-cell stage embryo with a fluorescent lineage marker (rhodamine-dextran) that allows identification of living neurons containing the constructs; myc staining demonstrated >90% correlation with fluorescently labeled cells, indicating that the constructs are expressed and that the proteins are stable over the time period examined. Injection of a construct encoding a myc-tagged 26 amino acid CN autoinhibitory peptide (Hubbard and Klee, 1989; Perrino et al., 1995) into one cell of two-cell stage embryos has no effect on neurite lengths in culture (data not shown).

In contrast, expression of a 97 amino acid fragment of the C terminal of CN (including the 26 amino acid autoinhibitory peptide), which has been shown to be eightfold more potent than

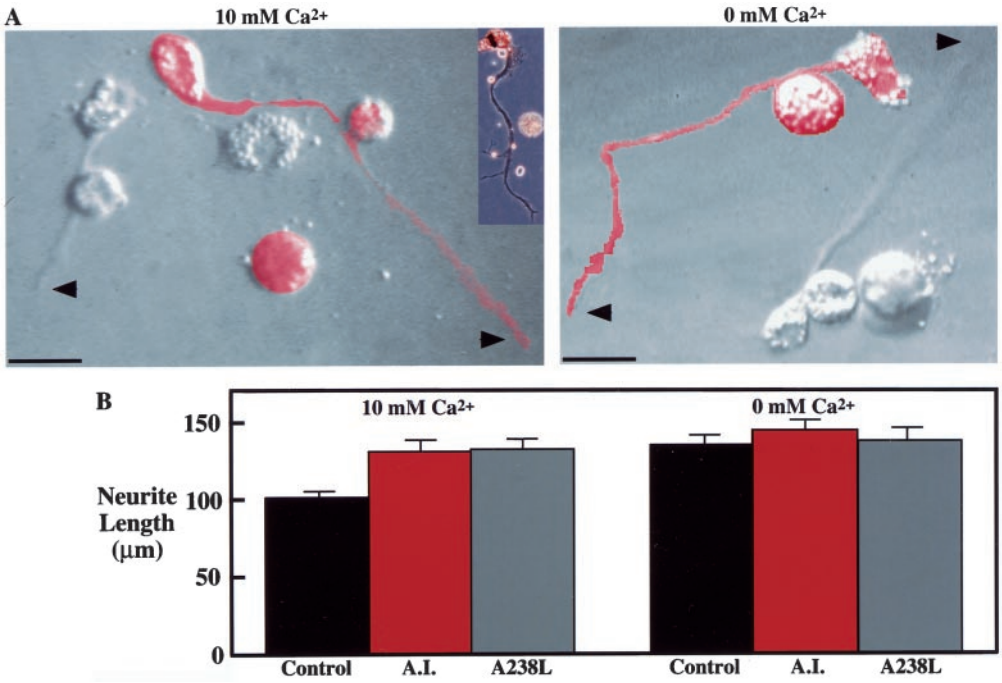

Figure 3. Peptide inhibition of CN increases neurite lengths in the presence of Ca^{2+}. *A,* Myc-tagged xCN autoinhibitory domain (*A.I.*) cRNA was coinjected into one cell of a two-cell stage embryo with a fluorescent lineage tracer (rhodamine-dextran) to identify *A.I.*-expressing cells. Nonfluorescent neurons serve as internal controls. *Arrowheads* indicate growth cones. *Inset* shows a myc-labeled neuron, demonstrating stability of the construct at 1 d in culture, is shown. Neurons were grown $\pm Ca^{2+}$. Scale bars, 25 μm. *B,* Neurite extension in the presence of external Ca^{2+} is significantly increased in cells expressing *A.I.* or the viral CN inhibitor A238L ($p < 0.01$). Neurons grown in the absence of Ca^{2+} and expressing *A.I.* or A238L have neurite lengths similar to those of controls. Data are mean ± SEM ($n \geq 60$).

the shorter peptide in inhibiting enzyme activity (Sagoo et al., 1996), leads to neurons with greater neurite lengths when grown in the presence of Ca^{2+} (Fig. 3*A*). Injecting 5–10 ng cRNA had no effect on neurite length, whereas increasing the amount to 20–25 ng promoted neurite extension in the presence of Ca^{2+} to lengths not significantly different from those seen in the absence of Ca^{2+} ($p < 0.01$). These results are consistent with the titration of endogenous xCN, and suggest that the effect of the autoinhibitory peptide is specific. The increases in neurite length by expression of the autoinhibitory peptide, CsA or DM treatment, or the absence of Ca^{2+} are indistinguishable (Fig. 3*B*; $p < 0.01$). Moreover CsA treatment of neurons expressing effective concentrations of autoinhibitory peptide yielded neurite lengths that were not different from those obtained by treatment with either inhibitor alone. Confirmation of the results with the autoinhibitory peptide was provided by experiments using a construct encoding myc-tagged African swine fever virus protein A238L, which has no sequence similarity to the autoinhibitory peptide and blocks CN activity by a different mechanism (Miskin et al., 1998). Again neurite extension is more rapid in neurons cultured in the presence of Ca^{2+} and achieves lengths similar to those of neurons grown in the absence of Ca^{2+} (Fig. 3*B*). Expression of either construct has no effect on neurite lengths in the absence of Ca^{2+} waves. These results with two specific peptide inhibitors of CN activity, with the convergent effects of the pharmacological inhibitors, suggest that this enzyme is a necessary component of the signal transduction cascade by which Ca^{2+} regulates neurite outgrowth.

Constitutively active calcineurin restricts neurite outgrowth

To resolve whether xCN is sufficient to regulate neurite extension, a CA form of xCN was created to test for gain of function in the absence of extracellular Ca^{2+}. Using an approach similar to that for construction of the autoinhibitory peptide, we generated a myc-tagged truncated form of CN containing the catalytic region of the enzyme but lacking the calmodulin-binding and autoinhibitory domains to produce a Ca^{2+}-independent enzyme. CA-xCN A cRNA was coinjected into one cell of a two-cell stage embryo with murine CN B cRNA (O'Keefe et al., 1992) to enhance A subunit stability. The mouse CN B subunit is 100% identical to the rat, bovine, and human homologs at the amino acid level (C. E. Nargang, A. D. Bottorff, and K. Adachi, GenBank accession number GI180705, 1993) (Guerini et al., 1989; Ueki et al., 1992), implying that murine CN B is a sufficient substitute for *Xenopus* CN B. A fluorescent lineage marker (FITC-dextran) was coinjected with CA-xCN A and murine CN B to identify expression in living neurons. Neurite extension is inhibited in the absence of external Ca^{2+} in neurons expressing the CA-xCN enzyme, yielding neurite lengths similar to those observed for neurons grown in the presence of Ca^{2+} and not expressing the construct (Fig. 4; $p < 0.01$). Expression of CN B alone or with an inactive form of CA-xCN (linked to green fluorescent protein at the C terminal) is ineffective in restricting neurite lengths. Neurons expressing CA-xCN and grown in the presence of Ca^{2+}, enabling production of Ca^{2+} waves, have neurite lengths that are not different from controls (Fig. 4*B*). This result indicates that a constitutively active form of CN mimics the effect of Ca^{2+} waves. The effect of CA-xCN is predicted to be inhibited by CsA (Clipstone et al., 1994) because it contains the site to which this agent binds. Indeed, addition of CsA to neurons expressing CA-xCN negates the inhibitory effect of CA-xCN and increases neurite lengths to an extent not significantly different from that observed in the absence of Ca^{2+} ($p < 0.01$). The reciprocal effects of xCN loss and gain of function in the presence and absence of Ca^{2+} waves suggest that this Ca^{2+}-dependent enzyme is necessary and sufficient to regulate neurite extension in response to transient elevations of Ca^{2+}.

Developmental expression of *Xenopus* calcineurin

To characterize the developmental appearance of xCN, we performed whole-mount *in situ* hybridization using albino *Xenopus* embryos over a range of developmental stages to determine its spatial and temporal expression pattern. Using a 1.2 kb antisense

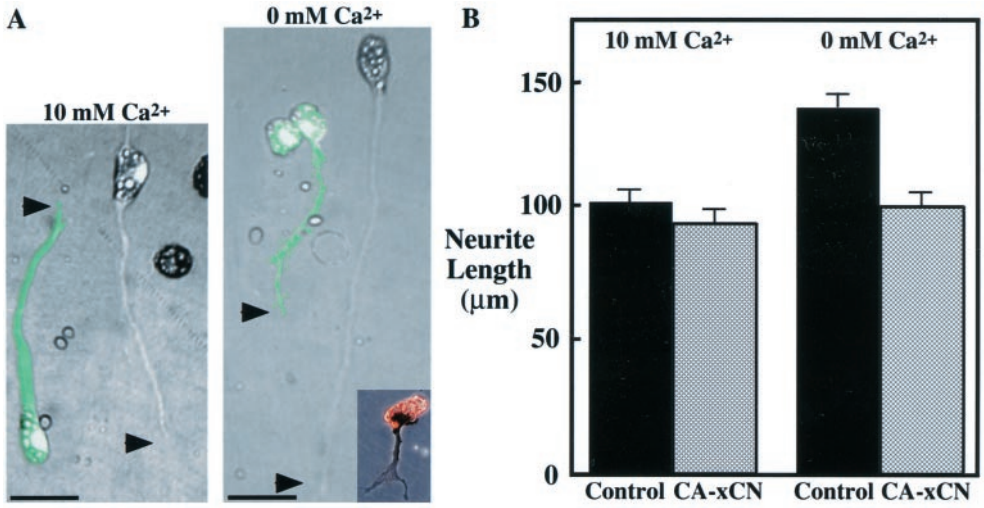

Figure 4. Expression of a constitutively active xCN construct leads to short neurites in the absence of Ca^{2+}. *A,* Myc-tagged CA-xCN A cRNA was coinjected into one cell of a two-cell stage embryo with mouse CN B cRNA to enhance enzymatic activity and a fluorescent lineage tracer (FITC-dextran) to identify CA-xCN-expressing cells. Nonfluorescent neurons are internal controls. *Arrowheads* indicate growth cones. *Inset* shows a myc-labeled neuron at 1 d in culture is shown. Neurons were grown $\pm Ca^{2+}$. Scale bars, 25 μm. *B,* Neurite extension is significantly inhibited in neurons expressing CA-xCN in the absence of external Ca^{2+} ($p < 0.01$). Neurons expressing CA-xCN grown in the presence of Ca^{2+} have neurite lengths similar to those of controls. Data are mean ± SEM ($n \geq 100$).

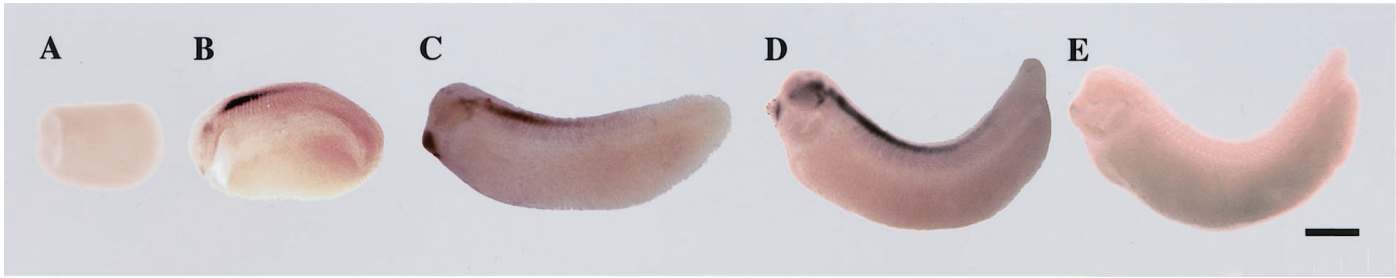

Figure 5. Developmental expression of *Xenopus* calcineurin transcripts is visualized by *in situ* hybridization of whole-mount albino *Xenopus* embryos with an xCN-specific antisense probe. *A,* xCN mRNA is not detected at the neural plate stage (stage 15). *B,* Message is first observed at the neural tube stage in the presumptive brain (stage 22). *C,* Transcripts are upregulated in the brain and spinal cord by the early tailbud stage (stage 26). *D,* Expression increases posteriorly as development progresses to the late tailbud stage (stage 32). *E,* A control tailbud stage embryo hybridized with xCN-specific sense probe does not reveal staining, indicating specificity of the antisense. For all embryos anterior is to the *left*, and dorsal is *up.* Scale bar, 500 μm.

fragment of the 3′ portion of the gene, we first detected xCN at the neural tube stage in the presumptive brain (Fig. 5). Transcripts are upregulated in the brain and spinal cord as development continues to tailbud stages, increasing from anterior to posterior. The expression pattern of xCN *in vivo* coincides with the Ca^{2+}-sensitive period of neurite extension observed *in vitro* and *in situ*, placing xCN in the correct place and at the right time to be involved in the signal transduction pathway through which Ca^{2+} waves control neurite extension.

Calcineurin lies downstream of Ca^{2+} waves

To determine whether CN regulates the production of waves, we measured the frequencies of Ca^{2+} waves in growth cones during both inhibition and activation of CN. Using the Ca^{2+} indicator dye Fluo 3 to measure changes in intracellular Ca^{2+}, we imaged growth cones for 30 min between 12 and 24 hr in culture (Fig. 6*A*). In the presence of CsA and DM at concentrations sufficient to increase neurite growth rates, spontaneous Ca^{2+} wave frequencies are not different from controls (Fig. 6*B*). Similarly, in neurons expressing CA-xCN identified by coexpression of fura-2-dextran, Ca^{2+} wave frequencies are not different from controls. Neither the pharmacological blockers nor the constitutively active construct affect the Ca^{2+} wave amplitude, duration, or time integral (data not shown). Because manipulating CN activity had no effect on Ca^{2+} wave frequency, we conclude that CN acts downstream of Ca^{2+} waves in controlling neurite extension.

Calcineurin does not regulate developmental expression of GABA

Ca^{2+} spikes have been shown to control GABA synthesis by regulating levels of transcripts of a gene encoding the synthetic enzyme glutamic acid decarboxylase (S. D. Watt, X. Gu, R. D. Smith, and N. C. Spitzer, unpublished results). Since CN has been shown to be involved in a variety of transcriptional control pathways, including those involved in cardiac development and disease (de la Pompa et al., 1998; Molkentin et al., 1998), the immune response (O'Keefe et al., 1992; Luo et al., 1996; Shibasaki et al., 1996; Beals et al., 1997; Chow et al., 1997), and neurotransmitter release (Wang and Kelly, 1997), Ca^{2+} spikes could also activate CN to control the expression of GABA. When neurons are grown in the absence of extracellular Ca^{2+}, spikes are abolished, and the incidence of GABA immunoreactivity drops to 10%, significantly lower than the 40% of neurons that are immunoreactive in the presence of external Ca^{2+}. To investigate whether CN is involved in the Ca^{2+}-dependent expression of GABA, we examined the extent of GABA immunoreactivity in the presence of pharmacological inhibitors of CN or the expression of myc-tagged CA-xCN. Neither application of CsA or DM nor expression of CA-xCN affected GABA immunoreactivity in the presence and absence of Ca^{2+} (Fig. 6*C*), showing that CN activity is not involved in the regulation of GABA synthesis by Ca^{2+} spikes.

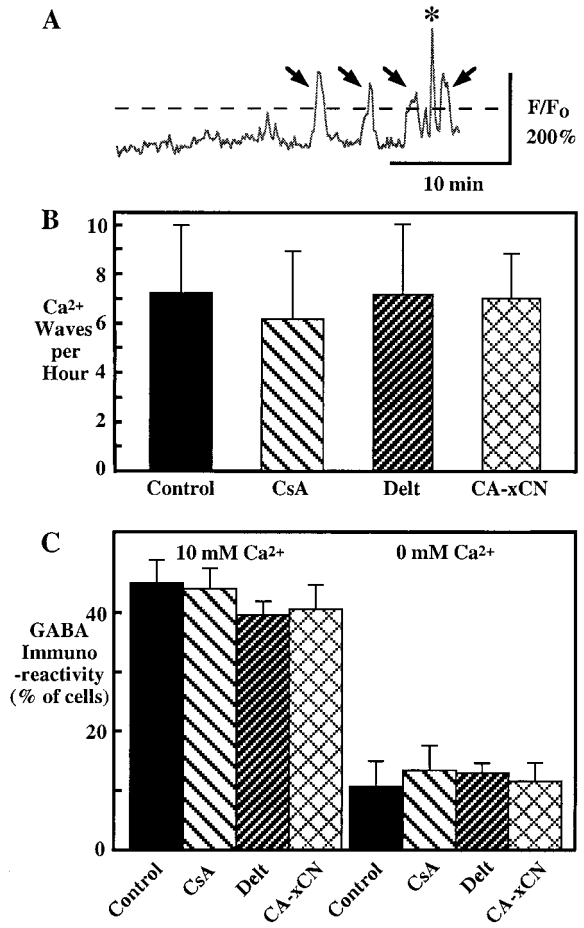

Figure 6. Ca^{2+} wave frequency and Ca^{2+}-dependent expression of GABA are not affected by inhibition or activation of CN. *A,* Spontaneous Ca^{2+} waves are shown. Intracellular Ca^{2+} was monitored using the Ca^{2+} indicator dye Fluo 3, and images were acquired at 10 sec intervals in the presence of 1 nM DM. *Arrows* indicate events scored as waves, the *asterisk* denotes a Ca^{2+} spike, and the *dashed line* indicates the event threshold. *B,* Inhibition of CN does not significantly affect the frequency of Ca^{2+} waves: control, 7 ± 3/hr; CsA (10 nM), 6 ± 3/hr; DM (*Delt*, 1 nM), 7 ± 3/hr. Neurons expressing CA-xCN, indicated by the fluorescence of fura-2-dextran, generate spontaneous Ca^{2+} waves at normal frequencies in growth cones: control, 7 ± 3/hr; CA-xCN, 7 ± 2/hr. Neurons were imaged for 30 min intervals. Data are mean ± SEM ($n \geq 15$). *C*, Neurons treated with either 10 nM CsA or 1 nM DM were assayed for expression of GABA with a rabbit anti-GABA polyclonal antibody. CN-inhibited neurons express the same extent of GABA immunoreactivity as controls. Reciprocal experiments with neurons expressing CA-xCN, confirmed by myc immunoreactivity, demonstrate no effect on GABA expression. Data are mean ± SEM ($n \geq 100$).

Ca^{2+} waves act via xCN to dephosphorylate GAP-43

Given the function of CN in regulating axon outgrowth, we sought to identify targets of its action that are controlled by wave activity. GAP-43 is a known substrate of CN that is concentrated in growth cones (Liu and Storm, 1989; Apel and Storm, 1992), inhibiting actin filament polymerization and slowing neurite outgrowth when dephosphorylated (He et al., 1997). By the use of antibodies that recognize phosphorylated GAP-43, high levels have been associated with growing neurites, whereas collapsing growth cones contain low levels of phosphorylated GAP-43 (Dent and Meiri, 1998).

To test the hypothesis that Ca^{2+} waves act via xCN to dephosphorylate GAP-43, we expressed rat GAP-43 in *Xenopus* neurons as an indicator of CN-dependent GAP-43 dephosphorylation. Using an antibody that specifically recognizes phosphorylated rat GAP-43 and does not cross-react with *Xenopus* GAP-43 (data not shown), we compared the extent of phosphorylated GAP-43 in the presence and absence of Ca^{2+} waves. Western blot analysis demonstrates that the level of phosphorylated GAP-43 increases in conditions in which Ca^{2+} waves are blocked and xCN activity is low (Fig. 7). Furthermore, suppression of CN activity with deltamethrin (1 nM) in the presence of Ca^{2+} increases the level of phosphorylated GAP-43 to an extent similar to that achieved by removal of extracellular Ca^{2+}. These findings suggest that GAP-43 phosphorylation is controlled by Ca^{2+} wave activity via xCN.

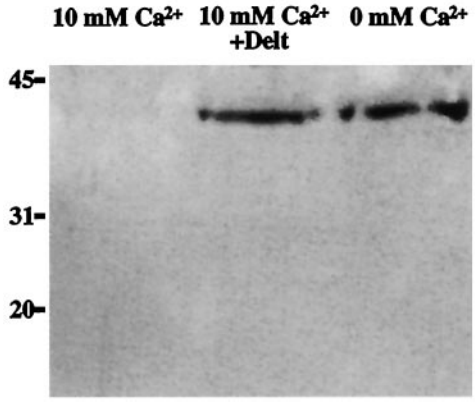

Figure 7. Ca^{2+} waves act via calcineurin to dephosphorylate GAP-43. A Western blot of extracts of cultured neurons grown in the presence of Ca^{2+} and Ca^{2+} + DM (*Delt,* 1 nM) and in the absence of Ca^{2+} demonstrates that GAP-43 phosphorylation is lower in the presence of Ca^{2+} and that inhibiting calcineurin returns this phosphorylation to levels similar to those observed in the absence of Ca^{2+} ($n = 7$).

Calcineurin regulates Ca^{2+}-dependent reorganization of the growth cone actin cytoskeleton

Microfilaments and microtubules are crucial cytoskeletal elements for growth cone motility (Lin and Forscher, 1993; Bentley and O'Connor, 1994; Challacombe et al., 1996) and are thus likely targets of Ca^{2+} transients. Dephosphorylation of GAP-43 by Ca^{2+} waves and CN suggested regulation of the actin cytoskeleton. If CN acts on both microfilaments and microtubules in the growth cone, as implied by its immunolocalization with these cytoskeletal components (Ferreira et al., 1993), disruption or stabilization of either of these components is expected to occlude partially the effects of blocking or stimulating CN activity. A dose–response analysis with cytochalasin D (Cyto D), which destabilizes actin filaments, identifies a critical concentration (50 nM) that does not lead to neurites that are shorter than those in the presence of Ca^{2+} (Fig. 8*A,B*; $p < 0.01$) or in the absence of Ca^{2+} for neurons expressing CA-xCN (Fig. 8*A*). However, in the presence of Cyto D, neurites grown in the absence of Ca^{2+} are as short as those observed in the presence of Ca^{2+} waves, suggesting that waves act to destabilize filamentous actin (Fig. 8*A*; $p < 0.01$). As expected, Cyto D also overrides the growth-promoting effect of inhibiting CN by CsA in the presence of Ca^{2+} and results in shortened neurite lengths. These results suggest that the enhancement of neurite outgrowth produced in the absence of waves or by suppression of CN is caused by increased dynamic assembly of actin filaments. Because the ef-

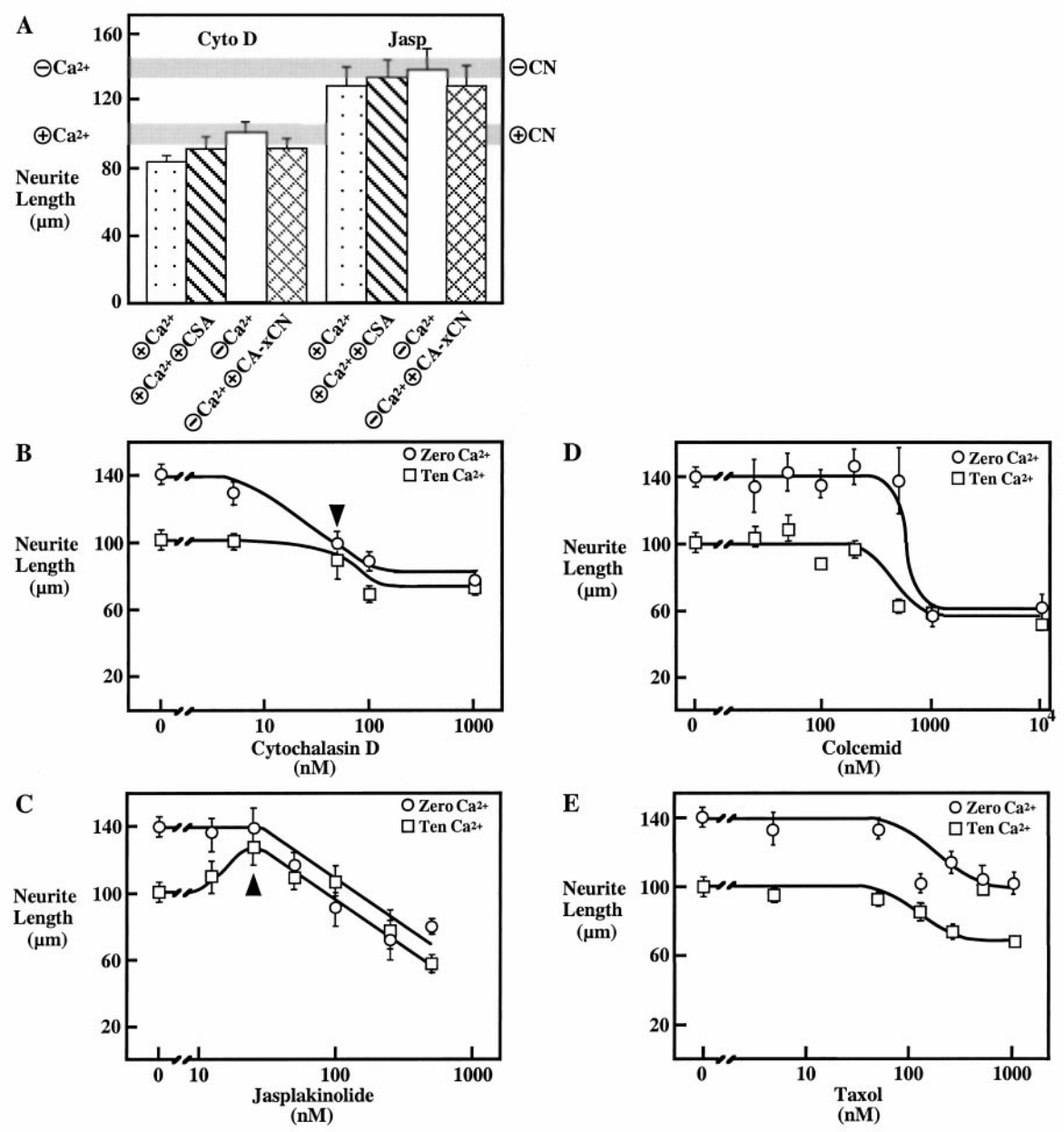

Figure 8. Disruption or stabilization of actin mimics the effects of activation or inhibition of CN. *A, Shaded horizontal bars* indicate neurite lengths in the presence of Ca^{2+} and CN activity and in their absence (see Fig. 1). Disruption of actin with Cyto D (50 nM) results in shorter neurites in the absence of CN activity, whereas stabilization of actin with Jasp (25 nM) generates longer neurites in the presence of CN activity. See text for further details. *B*, *C,* Dose–response analyses reveal the concentrations of Cyto D and Jasp (*arrowheads*) that mimic the effect of activating or inhibiting CN on neurite outgrowth. Disruption of actin filaments with Cyto D (50 nM; *B*) inhibits outgrowth in the absence of Ca^{2+} but not in its presence, whereas Jasp (25 nM; *C*) increases neurite lengths in its presence of Ca^{2+} but not in its absence. *D*, *E,* Dose–response analyses demonstrate that destabilization or stabilization of microtubules with colcemide (*D*) or taxol (*E*) results in a Ca^{2+}-independent inhibition of neurite outgrowth. Data are mean ± SEM ($n \geq 30$). *CSA*, CsA.

fects of Ca^{2+} waves and CN activity and actin destabilization are not additive, CN seems to act directly or indirectly on actin to control neurite extension.

In complementary experiments, stabilization of actin filaments with jasplakinolide (Jasp) (25 nM) (Bubb et al., 1994) in the presence of Ca^{2+} waves and CN activity results in neurite lengths similar to those seen in the absence of waves (Fig. 8*A*; $p < 0.01$). At higher concentrations of Jasp (>50 nM), neurite growth is inhibited (Fig. 8*C*), probably because of overstabilization of actin filaments. Jasp does not promote additional elongation of neurites in the absence of Ca^{2+} waves or when CN activity is blocked with CsA in the presence of Ca^{2+}. Jasp also negates the growth-inhibiting effects of CA-xCN in the absence of Ca^{2+} and leads to longer neurites. These results demonstrate that increased stabilization of actin promotes neurite growth in a manner similar to that seen in the absence of Ca^{2+} waves and CN activity and suggest that actin stability is correlated with neurite extension in a Ca^{2+}- and CN-dependent manner.

To ascertain the role of microtubules in Ca^{2+}- and CN-dependent neurite growth, we tested the effects of microtubule-

destabilizing (colcemide) and -stabilizing (taxol) agents on neurite lengths in the presence and absence of Ca^{2+} and CN activity (Fig. 8*D,E*). Neurite lengths are inhibited to a similar extent in the presence or absence of Ca^{2+} by colcemide (10 μM; 51%, $+Ca^{2+}$; 47%, $-Ca^{2+}$; $n > 30$) and taxol (1 μM; 75%, $+Ca^{2+}$; 77%, $-Ca^{2+}$; $n > 30$). These compounds do not appear to suppress production of Ca^{2+} waves, because they do not stimulate greater neurite lengths in the presence of Ca^{2+} at any concentration tested. The absence of a Ca^{2+}-dependent effect of microtubule stabilization or disruption on neurite elongation suggests that this cytoskeletal component is not involved in the pathway regulated by Ca^{2+} waves. This result is consistent with regulation of the advance of microtubules in growth cones by actin filaments (Lin and Forscher, 1995; Letourneau, 1996), obviating a requirement for independent regulation by Ca^{2+} and CN activity.

DISCUSSION

Calcineurin decodes Ca^{2+} waves to control neurite extension

We report that CN is required for regulation of neurite outgrowth by spontaneous Ca^{2+} waves in cultured *Xenopus* spinal neurons. In the presence of extracellular Ca^{2+}, enabling production of spontaneous waves, suppression of CN activity either pharmacologically or with an inhibitory peptide or protein results in longer neurites similar to those of neurons grown in the absence of waves. Slowing of neurite outgrowth by imposed Ca^{2+} transients is suppressed by CsA, further supporting the conclusion that the effect of spontaneous Ca^{2+} waves is mediated via CN. Conversely, when a constitutively active construct (CA-xCN) is expressed in neurons not generating Ca^{2+} waves, neurites are shorter and similar in length to those generating Ca^{2+} waves. This observation in conjunction with the absence of inhibition in the presence of extracellular Ca^{2+} argues that there are few if any spurious effects of its expression. However we cannot exclude the possibility that CA-xCN expression has effects before neurite extension. Previous pharmacological studies implicate CN in axonal regeneration (Lyons et al., 1994; Chang et al., 1995), but more recent reports suggest that immunophilins are the principal agents in some cases (Gold, 1997; Snyder et al., 1998). Our observations of the effect of DM and the CN autoinhibitory peptide, which block CN by mechanisms that do not involve immunophilins (Enan and Matsumura, 1992; Sagoo et al., 1996), and the absence of an effect of Rap, which binds to immunophilins but does not block CN (Liu et al., 1991), support a role for CN in Ca^{2+}-dependent neurite extension. Our results suggest that Ca^{2+}-dependent regulation of neurite length is not attributable to other serine–threonine phosphatases. Because xCN is expressed in the spinal cord of the developing embryo during the time when axon outgrowth is occurring (Gomez and Spitzer, 1999), it is likely to regulate neurite extension and pathfinding *in vivo*. CN activity does not affect the natural frequency of Ca^{2+} waves in growth cones, placing CN downstream of waves in the Ca^{2+}-dependent cascade that controls neurite elongation. We also demonstrate that CN is not the only Ca^{2+}-transient sensor in *Xenopus* neurons, because inhibition of endogenous xCN and expression of CA-xCN do not affect a second Ca^{2+}-dependent pathway by which spontaneous Ca^{2+} spikes control expression of GABA.

Wave activity controls GAP-43 phosphorylation levels via CN, suggesting that xCN acts via GAP-43 to regulate cytoskeletal actin structure and neurite length. Removing the contribution of the actin network to neurite extension with Cyto D or Jasp reveals the effect of Ca^{2+} on microtubules in neurite outgrowth and shows that at high concentrations of Cyto D and Jasp there is no difference in neurite outgrowth $\pm$ Ca^{2+}. Thus Ca^{2+} elevations seem to have little effect on microtubules. Conversely, when the contribution of microtubule dynamics to neurite outgrowth is removed by colcemide or taxol, the Ca^{2+}-dependent effect on actin is apparent. Stabilization of microtubules with taxol does not affect Ca^{2+}-dependent regulation of neurite length. However, when microtubules are destabilized by colcemide, neurites are inhibited similarly $\pm$ Ca^{2+}, presumably because the actin network is dependent on a microtubular scaffold. These experiments support the hypothesis that Ca^{2+} transients that act to destabilize the actin network have substantially less effect on microtubules. The results are consistent with observations that Ca^{2+} suppresses growth cone motility in chick dorsal root ganglion neurons (Lankford and Letourneau, 1989) and that elevation of Ca^{2+} is mimicked by low doses of cytochalasins (Lankford and Letourneau, 1991). The activity of CN seems to be both necessary and sufficient to suppress the organization of the growth cone actin cytoskeleton associated with rapid neurite outgrowth. Disruption or stabilization of the actin cytoskeleton occludes the effects of altering the activity of CN.

A model for CN regulation of neurite length

CN is an abundant Ca^{2+}- and calmodulin-dependent protein phosphatase, comprising ~1% of the total protein in brain (Klee et al., 1988). Its widespread expression underscores the many roles it plays in a variety of signal transduction cascades (Yakel, 1997), and it has been implicated in transcriptional control of numerous signaling pathways. However the effects of local inactivation of CN (Chang et al., 1995) and the ability of isolated growth cones to respond to Ca^{2+} elevations (Rehder and Cheng, 1998) both make it unlikely that activation of CN via Ca^{2+} influx

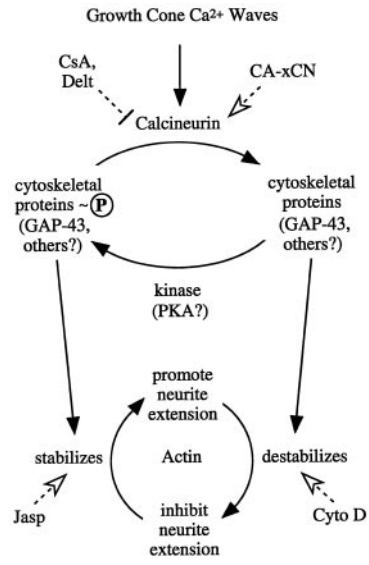

Figure 9. Model of the signal transduction cascade by which Ca^{2+} waves act via CN to regulate neurite extension. Ca^{2+} waves activate CN, shifting cytoskeletal-regulating proteins to a dephosphorylated state, destabilizing actin filaments, and inhibiting neurite elongation. CN is not activated in the absence of Ca^{2+} waves, shifting cytoskeletal-regulating proteins to a phosphorylated state, stabilizing actin filaments, and promoting neurite outgrowth. Activating or inhibiting this cascade at different points increases or decreases neurite extension. *Delt*, DM.

influences local cytoskeletal reorganization via a transcriptional mechanism. Activation of CN by Ca^{2+} transients promotes migration in neutrophils (Lawson and Maxfield, 1995), in contrast to the inhibition of neurite outgrowth reported here. Thus a single enzyme can have different effects depending on other components of the signal transduction cascade in which it functions.

Regulation of neurite extension by the phosphorylation state of cytoskeletal components in the growth cone raises the question of the identity of the reciprocal kinase that acts in concert with CN to promote the outgrowth of neurites. CN is associated with PKA and PKC via AKAP79, a common anchoring protein in neurons, which suggests that these two kinases act on common substrates to control their phosphorylation state (Coghlan et al., 1995; Klauck et al., 1996). PKC does not seem to play a role in regulation of neurite extension in *Xenopus* spinal neurons, because suppression and stimulation of its activity with different concentrations of phorbol 12-myristate 13-acetate (PMA) have no effect on neurite outgrowth (Gu and Spitzer, 1995). These findings, together with preliminary evidence that neurite lengths are longer when *Xenopus* spinal neurons are grown in the presence of 8-bromo-cAMP (Y. Gorbunora and N. C. Spitzer, unpublished observations), make PKA an attractive candidate in a reciprocal model of growth cone phosphorylation levels (Fig. 9). Furthermore, PKA has been shown to phosphorylate GAP-43 in cultures of rat striatum (Schmidt et al., 1998), and regulation of neurite outgrowth by CN and PKA has been described for cultured chick dorsal root ganglion neurons (Letourneau, 1996). Additionally this model provides a mechanism that allows independent stimulation and inhibition of neurite outgrowth.

Although CN activity inhibits neurite elongation by its effect on the actin cytoskeleton, many other components are involved in promoting Ca^{2+}-independent neurite outgrowth. For example, microtubule-associated protein (MAP)-1B regulates microtubule bundling, cross-linking of microtubule and actin filaments, and axon extension in a phosphorylation-dependent manner (Gordon-Weeks, 1993; Avila et al., 1994; Ulloa et al., 1994). A substantial increase in MAP-1B phosphorylation occurs in differentiating neurons (Diaz-Nido et al., 1990) and may be required to assemble a stable microtubule scaffold (Denoulet et al., 1989; Diaz-Nido et al., 1990). MAP-1B is phosphorylated by casein kinase or proline-directed protein kinases (Diaz-Nido et al., 1990; Avila et al., 1994), but the phosphatase that dephosphorylates it is presently unknown. Thus multiple mechanisms converge to regulate axon extension in embryonic neurons.

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