Research Report

Laminin activates CaMK-II to stabilize nascent embryonic axons

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ABSTRACT

In neurons, the interaction of laminin with its receptor, β1 integrin, is accompanied by an increase in cytosolic Ca2+. Neuronal behavior is influenced by CaMK-II, the type II Ca2+/calmodulin-dependent protein kinase, which is enriched in axons of mouse embryonic neurons. In this study, we sought to determine whether CaMK-II is activated by laminin, and if so, how CaMK-II influences axonal growth and stability. Axons grew up to 200 μm within 1 day of plating P19 embryoid bodies on laminin-1 (EHS laminin). Activated CaMK-II was found enriched along the axon and in the growth cone as detected using a phospho-Thr287 specific CaMK-II antibody. β1 integrin was found in a similar pattern along the axon and in the growth cone. Direct inhibition of CaMK-II in 1-day-old neurons immediately froze growth cone dynamics, disorganized F-actin and ultimately led to axon retraction. Collapsed axonal remnants exhibited diminished phospho-CaMK-II levels. Treatment of 1-day neurons with a β1 integrin-blocking antibody (CD29) also reduced axon length and phospho-CaMK-II levels and, like CaMK-II inhibitors, decreased CaMK-II activation. Among several CaMK-II variants detected in these cultures, the 52-kDa δ variant preferentially associated with actin and β3 tubulin as determined by reciprocal immunoprecipitation. Our findings indicate that persistent activation of δ CaMK-II by laminin stabilizes nascent embryonic axons through its influence on the actin cytoskeleton.

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1. Introduction

Changes in intracellular Ca2+ elicit profound effects on the behavior of developing neurons, particularly at the growth cone (Bolsover, 2005; Henley and Poo, 2004). Changes in Ca2+ can be modulated by location, amplitude, and timing to differentially influence gene expression, neurite morphogenesis, and axon guidance (Gomez and Spitzer, 2000; Spitzer et al., 2000). One of many potential Ca2+ targets is CaMK-II, a Ca2+ and CaM(calmodulin)-dependent protein kinase, which is known to influence neurite extension, arborization, and dynamics (Fink et al., 2003; Goshima et al., 1993; Johnson et al., 2000; Massé and Kelly, 1997; Nomura et al., 1997; Tashima et al., 1996; Wen et al., 2004; Zou and Cline, 1996), most likely through its effects on the actin cytoskeleton (Caran et al., 2001; Fink et al., 2003; Shen and Meyer, 1999; Shen et al., 1998). CaMK-II expression increases in cultured hippocampal neurons and in differentiating P19 embryonic neurons, where at least one β and three δ CaMK-II variants are expressed (Donai et al., 2000; Fink et al., 2003; Johnson et al., 2000; Scholz et al., 1988). In P19 cultures, the majority of δ CaMK-II is axonal (Faison et al., 2002); however, little is currently known about

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the stimuli which lead to the Ca\textsuperscript{2+} flux necessary to activate this axonal CaMK-II. In addition, neither the substrates of CaMK-II nor their mode of influence on axon growth or stability is known.

Laminin is an extracellular matrix protein that promotes neurite outgrowth and axonal specification (Hynds and Snow, 2001; Kuhn et al., 1998; Liesi et al., 2001; Matsuzawa et al., 1996; Powell et al., 1998, 2000; Tang and Goldberg, 2000; Tomaselli et al., 1993). Laminin-1 (EHS laminin) is the principal laminin involved in epithelial morphogenesis, preneuronal cell migration and neurite outgrowth (Ekblom et al., 2003; Miner and Yurchenco, 2004). Laminins influence cell behavior through integrins, their cell-surface receptor. Integrins not only mediate the adhesion of cells to the extracellular matrix but initiate signals for an array of cellular functions (Hynes, 2002). Integrins are heterodimers; of the 24 different α-β combinations currently known, at least 12 contain the β\textsuperscript{1} subunit (Hynes, 2002). In differentiating P19 neurons, β\textsuperscript{1} integrin is the primary laminin receptor, exists as an α\textsubscript{5}β\textsuperscript{1} integrin complex, and like CaMK-II, increases in mass as neurons form (Dedhar et al., 1991).

Integrin activation by laminin leads to the elevation of intracellular Ca\textsuperscript{2+} in chick ciliary ganglion neurons (Bixby et al., 1994) and in dorsal root ganglion (DRG) neurons in association with growth cone turning (Kuhn et al., 1998). In migrating growth cones of Xenopus spinal cord neurons, β\textsuperscript{1} integrin sites of adhesion are sites of Ca\textsuperscript{2+} influx (Gomez et al., 2001). CaMK-II has been implicated as an important target of laminin-induced Ca\textsuperscript{2+} increases in DRG neurons (Kuhn et al., 1998) and is necessary for the integrin-induced elaboration of the Drosophila neuromuscular junction (Beumer et al., 2002).

Therefore, in this study, we examined whether laminin is the extracellular ligand, which leads to the activation of CaMK-II in de novo forming P19 axons. CaMK-II activation is absolutely dependent upon Ca\textsuperscript{2+}, but we did not characterize the relative contribution of internal and external Ca\textsuperscript{2+} sources. Rather, we examined the influence of β\textsuperscript{1} integrin-blocking antibodies on axon growth and endogenous CaMK-II activity by both biochemical and immunological approaches. We also examined whether CaMK-II activity influenced axonal growth or axonal stability. Our findings support a mechanism by which CaMK-II is activated by laminin in the developing axon to locally stabilize the cytoskeleton.

2. Results

2.1. Laminin accelerates attachment and neurite outgrowth

Neurons were prepared for this study from P19 cells by retinoic acid induction via embryoid body formation (McBurney et al., 1982). P19 cells are an ideal model system for the de novo formation of neurons, which rapidly form without feeder cell layers and express neocortical or hippocampal markers (Bain et al., 1994; Finley et al., 1996; Magnuson et al., 1995; Morassutti et al., 1994; Staines et al., 1994). Embryoid bodies attached immediately to laminin-coated dishes but tenuously to dishes pretreated with poly-L-lysine or left untreated. In the absence of laminin, neurite outgrowth was delayed for at least 24 h (Fig. 1A) but was observed within 2 h when embryoid bodies were plated on laminin. On laminin, neurites grew rapidly, reaching several hundred μm in length by 24 h (Figs. 1B, C).

Both attachment and outgrowth demonstrated a dose dependency on laminin, saturating at approximately 2 μg/ml.

2.2. β1 integrin and activated CaMK-II are found along P19 axons and in growth cones

β1 Integrin, which is the receptor for laminin in P19 neurons, was localized in cultures after 24 h of growth on laminin using an anti-β1 integrin antibody (CD29). β1 Integrin was concentrated in streaks in glial cells within the population (Fig. 2A) and along the axon shaft in neurons (Fig. 2B). Within the growth cone, β1 integrin was concentrated through the neck region with punctate distribution at the periphery (Fig. 2B).

Upon natural activation by Ca\textsuperscript{2+}/CaM, CaMK-II autophosphorylates on Thr\textsuperscript{287} and becomes Ca\textsuperscript{2+} independent (for review, see Hudmon and Schulman, 2002). Antibodies against this form of CaMK-II (phospho-Thr\textsuperscript{287}) can localize activated CaMK-II but do not distinguish between the known β and δ

Fig. 1 – P19 neurons cultured on laminin extend neurites immediately. P19 embryoid bodies were plated on dishes precoated with laminin-1 at 0 (A), 1.25 (B) or 2.5 μg/ml (C). Cells were photographed after 24 h in phase contrast. Scale bar represents 100 μm.
CaMK-IIIs expressed in these cultures (Donai et al., 2000; Johnson et al., 2000), which have a common sequence in this Thr²⁸⁷ region (Tombes et al., 2003). Activated CaMK-II was enriched along axons and was also present at lower levels at the tips of growth cones (Fig. 2C). The distribution of activated CaMK-II was similar to that of β₁ integrin along the axon and in the growth cone. Fixative incompatibility prohibited simultaneous staining for activated CaMK-II and β₁ integrin.

Activated CaMK-II was found along axons in colocalization with the neuronal-specific marker β₃ tubulin (Fig. 3A) but exhibited a less precise colocalization with F-actin, which was concentrated in the growth cone (Fig. 3B). Activated CaMK-II

Fig. 2 – β₁ Integrin and active CaMK-II colocalize in P19 neurons. P19 embryoid bodies plated on laminin were methanol-fixed after 24 h and stained with the CD29 anti-β₁ integrin antibody (A, B). 24-h cultures were also fixed with formaldehyde and stained with the anti-phospho-Thr²⁸⁷ CaMK-II antibody (C) or with preimmune serum (D). Images in phase contrast (top) and in fluorescence (bottom) are shown. Scale bar represents 10 μm.

Fig. 3 – Active CaMK-II colocalization with the cytoskeleton in P19 neurons. One-day-old neurons grown on laminin, fixed in formaldehyde and costained with the phospho-Thr²⁸⁷ CaMK-II antibody (P-CaMK-II) and anti β₃-tubulin (top middle) or phalloidin (bottom middle) were merged into a composite of phospho-CaMK-II (red) and β₃-tubulin or F-actin (green) to reveal colocalization (Composite). Scale bar represents 25 μm.
was greatly enriched along axons when compared to non-neuronal cells in the same culture (Fig. 3B).

2.3. **β₁ integrin-blocking antibody and CaMK-II inhibitors cause axon retraction**

Neither the presence of naturally activated CaMK-II along growing axons nor previous studies which inhibited CaMK-II prior to neuronal induction (Johnson et al., 2000) distinguish between a role for CaMK-II in stabilizing or in promoting the growth of axons. Therefore, in this study, neurons were grown for 1 day and then treated with CD29 and two compounds which act immediately, but differently, to inhibit CaMK-II. CD29 blocks laminin binding to β₁ integrin (Suzuki and Takahashi, 2003), as demonstrated by the complete inhibition of embryoid body attachment to laminin-coated dishes after preincubation at 10 μg/ml (data not shown). CaMK-II can be directly inhibited by KN-93, which is an antagonist of CaM binding in a select group of CaM kinases (Sumi et al., 1991). CaMK-II is half-maximally inhibited by KN-93 at 2–5 μM (Tombes et al., 1995) and is the principal target of KN-93 in P19 neurons since CaMK-I was undetectable by either immunoblotting or immunostaining (data not shown). CaMK-II was also directly inhibited by a myristoylated autoinhibitory peptide (myr-AIP), which is specific for CaMK-II and blocks activity at concentrations similar to KN-93 (Wen et al., 2004). This membrane permeant peptide directly interferes with catalysis by mimicking the CaMK-II autoinhibitory domain.

Direct CaMK-II inhibition for 2 h with 5 μM KN-93 or 5 μM myr-AIP resulted in axonal shortening and decreased CaMK-II activation, as detected using the anti-phospho-Thr287 antibody (Figs. 4B, C). Neurons treated with 10 μg/ml CD29 also retracted and exhibited reduced CaMK-II activation (Fig. 4D) when compared to untreated neurons (Fig. 4A).

Axon retraction in response to β₁ integrin inhibition was directly observed using time-lapse microscopy (Fig. 5 and supplement). KN-93 was used because it is readily reversible (Tombes et al., 1995). Phase contrast images acquired prior to treatment (0), 60 min after treatment with 5 μM KN-93 (70) and then 60 and 120 min after washout (140 and 200) are shown. Upon treatment, there was an immediate cessation of the normal dynamic behavior of the axon, as previously reported for KN-93 (Fink et al., 2003). Filopodial protrusions disappeared and the axon retracted. After washout, protrusive behavior along the axon was restored as the growth cone reformed and slowly re-extended. Retraction was maximal if CaMK-II was inhibited for at least 2 h at 37 °C.

Axon shortening as a result of β₁ integrin and CaMK-II antagonism was quantitatively determined after staining with the β₃ tubulin antibody, TUJ1. Axon lengths were measured in at least 50 cells per condition. β₃ Tubulin is expressed strongly along one or two extensions (Fig. 6A). Axons were significantly shortened if neurons were formed on laminin for 22 h and then treated for 2 h with KN-93, myr-AIP or CD29 (Fig. 6). Likewise, if neurons were cultured for 2 h and then treated for 22 h, axons were similarly shortened. Much like the effects observed on live neurons (Fig. 5), this result is consistent with a role for CaMK-II in axon stabilization rather than in axon growth. A representative image of a culture treated with myr-AIP shows shortened axons (Fig. 6B). KN-92, an inactive analog of KN-93 (Tombes et al., 1995), had no effect on axon length (Fig. 6). TUJ1 mouse IgG was also ineffective when added to cultures, demonstrating the specificity of the CD29 antibody (Fig. 6).

2.4. **β₁ integrin blockade prevents natural CaMK-II activation**

CD29 inhibited the natural activation of endogenous CaMK-II as measured through in vitro assays (Fig. 7). Since CaMK-II becomes Ca²⁺-independent (autonomous) when it autophosphorylates at Thr²⁸⁷, this assay quantitatively assesses that degree of activation as the percentage of the total (Ca²⁺-dependent) activity that is Ca²⁺-independent (percent...
For this assay, neurons were grown on laminin for 22 h, treated for 2 h, harvested and lysates were prepared. Although assays of cell lysates are reflective of the entire culture, activated CaMK-II is predominantly neuronal (Fig. 3). CaMK-II activation in untreated P19 neurons was higher (7.3%) than in uninduced P19 cells (3.2%), a result consistent with the natural activation of CaMK-II during neuronal induction. Activation levels at 10% or below are similar to those seen in other cell cultures (Tombes et al., 1999). CD29 was as effective at decreasing the level of CaMK-II activation as was direct CaMK-II inhibition by KN-93 and myr-AIP. These findings support the conclusion that β1 integrin bound to laminin naturally activates CaMK-II.

2.5. CaMK-II inhibition disorganizes F-actin

A role for CaMK-II in axon stabilization suggests effects on the cytoskeleton. Axons that retracted in response to CaMK-II antagonists and anti-integrin exhibited disassembled axonal microtubules (Fig. 6) and microfilaments. However, limited treatments for shorter times or at lower concentrations caused F-actin disorganization prior to disassembly (Fig. 8). Whereas untreated cultures showed an enrichment of well-organized F-actin in the growth cone (Fig. 8A), neurons treated for only 1 h with myr-AIP or KN-93 exhibited subtle changes in the distribution of F-actin. Actin filaments were still existent but were splayed in the growth cone with spurious F-actin seen along the axon (Figs. 8B and C). Partial reformation of growth cones with F-actin enrichment occurred 2 h after KN-93 washout (Fig. 8D).

2.6. δ CaMK-II binds to actin and β3 tubulin

Because CaMK-II is activated by laminin to influence the axonal cytoskeleton, and both β and δ CaMK-IIs have been reported to associate with actin, we examined whether endogenous β or δ CaMK-II coassociated with integrin, actin,
or tubulin. Both $\beta$ and $\delta$ CaMK-II immunoprecipitates contained actin and $\beta_3$ tubulin (Fig. 9, lanes 1 and 2). $\delta$ CaMK-II consistently exhibited more coassociated actin and tubulin than did $\beta$ CaMK-II. These samples as well as actin and $\beta_3$ tubulin immunoprecipitates (lanes 3–6) were then probed with biotinylated CaM, which reacts equally well with all CaMK-IIs. The $\delta$ CaMK-II and the actin immunoprecipitates contained almost exclusively a 52-kDa CaM-binding polypeptide (lanes 3 and 5). The $\beta$ CaMK-II immunoprecipitate (lane 4) contained a trace of the 52-kDa band and a 62-kDa band, but primarily a 57-kDa band. The 52-kDa and the 57-kDa bands were also immunoprecipitated by the $\beta_3$ tubulin antibody. These three bands were the same sizes as those previously identified as CaMK-IIs in P19 cultures (Donai et al., 2000; Johnson et al., 2000). We conclude that the 52 kDa $\delta$ CaMK-II preferentially but not exclusively associates with actin and neuronal-specific $\beta_3$ tubulin over the other CaMK-IIs present in these cultures. Although CaMK-II has been reported to associate with integrin in human mammary epithelial cells (Suzuki and Takahashi, 2003), no $\beta_1$ integrin was detected in any CaMK-II immunoprecipitate when assessed by CD29 immunoblot. Likewise, CD29 immunoprecipitates contained no detectable CaMK-II activity (data not shown).

3. Discussion

We have found that endogenous CaMK-II is activated by laminin via $\beta_1$ integrin to stabilize de novo formed embryonic axons. We have reached this conclusion through the following observations: (1) interference of the laminin-$$\beta_1$$ integrin interaction and direct inhibition of CaMK-II causes retraction of 1-day-old axons; (2) activated CaMK-II exhibits the same localization as $\mu_1$ integrin along the axon and in the growth cone.

Such a linkage between integrin and CaMK-II is not universal but depends on cell type and context. For example, antibodies that block $\alpha_6$$\beta_1$ integrin function have no effect on laminin-dependent neurite outgrowth in dorsal root ganglion neurons but are inhibitory to neurite outgrowth from retinal ganglion neurons cultured on laminin (Tomaselli et al., 1993). Inhibition of CaM kinase(s) with KN-62 has no effect on neurite outgrowth from cerebellar neurons cultured on laminin but is effective in blocking neurite outgrowth from cerebellar neurons induced by fibroblast growth factor and the cell adhesion molecules L1, N-CAM, and N-cadherin (Williams et al., 1995). CaMK-I, not CaMK-II, supports neurite elaboration in cerebellar and hippocampal neurons, whereas CaMK-II influences dendritic arborization in hippocampal neurons during a limited embryonic window (Fink et al., 2003; Wayman et al., 2004; Wen et al., 2004). Use of the P19 system to study the role of integrins and CaMK-II in the de novo formation of axons has proved advantageous as it bypasses the influence of other cells or the synthesis of new extracellular matrix molecules when studying the earliest events of neurite outgrowth.

Relative roles of Ca$^{2+}$-dependent enzymes in the growth, stability, and dynamics of neurites remains controversial despite subcellular and tissue specificities of these regulators. In addition to CaMK-I and CaMK-II, substantiated targets of neurogenic Ca$^{2+}$ fluxes include the protease calpain, and the Ca$^{2+}$/CaM-dependent phosphatase, calcineurin (Conklin et al., 2005; Fink et al., 2003; Robles et al., 2003; Wayman et al., 2004; Wen et al., 2004). Migrating growth cones exhibit complex patterns of Ca$^{2+}$ signals, which may reflect spatially and temporally distinct targets within the same cell as growth cones migrate, pause, turn, and branch (Bolsover, 2005; Tang Fig. 7 – Anti-$\beta_1$ integrin inhibits CaMK-II activation. Embryoid bodies were cultured on laminin for 22 h, treated for 2 h and then harvested. CaMK-II enzymatic activity was measured in cell lysates of uninduced P19 cells and of induced P19 neurons after treatment with 5 $\mu$M myr-AIP, 5 $\mu$M KN-93, 5 $\mu$M KN-92, and 10 $\mu$g/ml of the CD29 anti-$\beta_1$ integrin antibody. Percent CaMK-II activation represents Ca$^{2+}$-dependent activity as a percentage of Ca$^{2+}$-independent activity.

Fig. 8 – CaMK-II inhibition disorganizes F-actin. F-actin was detected using rhodamine–phalloidin in representative fixed P19 neurons before (A), 1 h after 5 $\mu$M myr-AIP (B) and 5 $\mu$M KN-93 (C) treatment, and 2 h post-KN-93 washout (D). Scale bar = 50 $\mu$m.
et al., 2003). Unlike other Ca²⁺ targets, CaMK-II is an ideal decoder of complex Ca²⁺ signals as it autophosphorylates in proportion to Ca²⁺ transient amplitude and frequency, rendering itself Ca²⁺-independent to varying degrees and for various lengths of time after the Ca²⁺ stimulus has subsided (De Koninck and Schulman, 1998; Hudmon and Schulman, 2002; Soderling et al., 2003).

Even within the CaMK-II family, there are over three dozen different splice variants (Hudmon and Schulman, 2002; Lantsman and Tombes, 2005; Tombes et al., 2003), which differentially influence neurite outgrowth and behavior. Their relative roles cannot easily be sorted out, due to their ability to heterooligomerize (Hudmon and Schulman, 2002; Lantsman and Tombes, 2005; Tombes et al., 2003) and their coexistence within a single cell type. For example, β and α CaMK-IIs coexist in hippocampal neurons, but only β CaMK-II influences neuronal dynamics (Fink et al., 2003). α CaMK-II, which is not expressed during embryonic development, is the principal hippocampal CaMK-II, and inhibits neurite outgrowth when overexpressed in both PC12 cells and hippocampal neurons (Bayer et al., 1996, 1999; Menegon et al., 2002; Silva et al., 1992; Tashima et al., 1996). Neurite outgrowth in PC-12 and P19 cultures is supported by δ CaMK-II (Donai et al., 2000; Fink et al., 2003; Johnson et al., 2000), which like β, but unlike α or γ CaMK-II, colocalizes with F-actin (Caran et al., 2001; Fink et al., 2003; Lantsman and Tombes, 2005).

In the embryonic neurons studied here, δ CaMK-II preferentially interacts with actin and β3 tubulin over β CaMK-II and therefore is concluded to be the axonal regulatory CaMK-II. This may be due to the fact that δ CaMK-II, which represents either the δc or the δα isozyme (Tombes et al., 2003), is the predominant CaMK-II and coexists along axons (Faison et al., 2002) with the cytoskeleton. The βδ CaMK-II variant expressed here interacts with β3 tubulin, so it is at least partially neuronal, but it does not coassociate with actin. The actin-associating properties of β CaMK-II are thought to be due to variable domain sequences (Fink et al., 2003; Shen et al., 1998), which are missing in the βδ variant (Tombes et al., 2003). In contrast, since the 52-kDa δ CaMK-II has no unique variable domains, its interaction with actin and β3 tubulin cannot be dependent on these alternative sequences.

The findings presented here suggest that CaMK-II is an influential linker of microtubules and microfilaments along embryonic axons. CaMK-II is known to form stable associations with microtubules and to phosphorylate tau, an axonal microtubule-associated protein (MAP) involved in bundling and neurite extension (Vallano et al., 1985; Yamamoto et al., 2002). CaMK-IIs are well known for their influence on F-actin stability (Caran et al., 2001; Fink et al., 2003), but this is the first study, which shows the association of a specific CaMK-II variant with both actin and tubulin. Since axons appear to have a zone of CaMK-II-independent stability at the proximal end, the stabilization of axons by CaMK-II would apply only to the distal end, possibly through the stimulatory phosphorylation of anchoring or bundling proteins. Interestingly, tau is enriched at the distal end of axons (Black et al., 1996). There are also many actin-associated proteins along the axon and in the growth cone whose phosphorylation by CaMK-II could influence axonal stability (Bolsover, 2005; Dent and Gertler, 2003; Henley and Poo, 2004). We are currently investigating the natural substrates of CaMK-II that influence axonal cytoskeleton stability.

We have concluded that δ CaMK-II acts via the cytoskeleton to stabilize axons rather than to promote their growth. The findings presented here may reflect a mechanism by which Ca²⁺ transients acting through CaMK-II and balanced by phosphatases, affect subtle, local characteristics of the axonal cytoskeleton (Henley and Poo, 2004; Wen et al., 2004; Zheng, 2000). Transient and localized alterations in axonal stability, as mediated by CaMK-II, now represent a plausible and testable mechanism by which both growth cone turning and axon branching could be regulated by Ca²⁺ signals.

4. Experimental procedures

4.1. Cell culture of P19 embryonal carcinoma cells

The P19 mouse diploid cell line was derived from an embryonic day 7 (E7) embryo and can be induced to differentiate by retinoic acid via embryoid body formation (McBurney et al., 1982). Undifferentiated P19 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS). For the induction of differentiation, cells were cultured at 1 × 10⁶ cells/ml in DMEM, 5% FBS, 5 × 10⁻⁷ M all-trans-retinoic acid (ATRA) in bacteriological Petri dishes for 4 days (Yao et al., 1995). Induction yields embryoid bodies, which were then plated at 2–5 × 10⁶ cells/cm² on culture dishes. Dishes were precoated with EHS laminin (Invitrogen) in neurobasal medium containing N2 supplement (Invitrogen). Poly-L-lysine (Sigma Chemical, St. Louis, MO) pretreatment was at 0.01% for 1 h in dH₂O. The only culture
modification was that cytosine arabinoside, which suppresses the growth of undifferentiated cells, was not added after plating (Yao et al., 1995).

4.2. Antibodies

The anti β1 integrin antibody was the CD29 mouse monoclonal IgG (BD Biosciences, San Jose CA). Anti-β3 tubulin monoclonal IgG (TUJ1) was provided by Dr. Anthony Frankfurter, University of Virginia, Charlottesville, VA. Rhodamine- or Oregon Green-conjugated phalloidin (Molecular Probes, Eugene OR), rabbit anti-actin antibody (Sigma), mouse anti-β3 integrin antibody was obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and goat anti-α CaMK-II (Santa Cruz Biotechnology, Santa Cruz CA) were purchased. Rabbit anti-phospho-Thr287 CaMK-II (Upstate Biotechnology Inc., Lake Placid NY) worked well in formaldehyde-fixed cells, but not methanol-fixed cells, and reacted only weakly on immunoblots with extracts from P19 neurons. Goat anti-CaMK-I antibody was obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. All secondary antibodies were from Kierkegaard Perry Labs, Gaithersburg, MD.

4.3. Immunolocalization

Cells were fixed in methanol at −20 °C for 5 min for integrin staining. All other samples were fixed in 4% formaldehyde, phosphate-buffered saline (PBS), at 4 °C for 15 min. Formaldehyde-fixed cells were then permeabilized for 5 min in 0.1% NP40, PBS. All fixed cells were blocked for 30 min in Tris-buffered saline, pH 7.4, with 0.1% Tween 20 (TBST), containing 5% bovine serum albumin and 2% appropriate preimmune serum. Cells were then incubated in primary antibody at 2–5 μg/ml for 2 hrs in 2% BSA, TBST, washed three times in TBST, incubated in secondary antibody at 2 μg/ml in blocking solution for 1 h, washed three times in TBST, and imaged or stored in PBS at 4 °C until ready for imaging.

4.4. Immunoprecipitation

Exactly 100 μg of sonicated lysate protein was incubated with 1 μg of primary antibody overnight at 4 °C. This was immunoprecipitated with 1 μg of either biotinylated goat anti-mouse, donkey anti-goat or goat anti-rabbit IgG (Kierkegaard Perry Labs, Gaithersburg, MD), followed by streptavidin-magnespheres (Promega, Madison WI). Samples were washed three times with TBST, containing 0.1% NP-40, resuspended in SDS sample buffer, and processed for immunoblotting, as described above.

4.5. Immunoblotting and calmodulin overlay

Samples were separated on 7.5% or 10% polyacrylamide gels using the Mini-Protean II gel electrophoresis system (Bio-Rad, Hercules CA). Proteins were transferred to 0.45 μm nitrocellulose sheets for 1 h at 100 V and blocked with TBST, containing 5% BSA and 2% preimmune serum of the secondary antibody host for 1 h. Blots were then incubated overnight with primary antibodies at 1 μg/ml in 2% BSA/TBST. Blots were washed three times with TBST and incubated for 1 h with alkaline phosphatase-conjugated secondary IgG at 2 μg/ml in 2% BSA/TBST. Blots were washed three times with TBST and developed with 0.25 mg/ml each nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.1 M NaCl, 5 mM MgCl₂, pH 9.4. For calmodulin overlays, samples were transferred to nitrocellulose and then blocked with TBST containing 2 mM CaCl₂ (TBSTC) and 5% BSA for 30 min. Blots were incubated in TBSTC with 1 μg/ml biotinylated calmodulin (Gibco-BRL) overnight, washed three times in TBSTC for 10 min, and then incubated with alkaline phosphatase-conjugated streptavidin (2 μg/ml) for 2 h in TBSTC at 24 °C. Blots were washed three times and developed as described above with NBT/BCIP. This approach avoids cross-reaction with the IgG heavy chain when probing immunoprecipitates.

4.6. Quantitative imaging

Cells were digitally imaged on an Olympus IX-70 inverted fluorescent microscope controlled by Microsuite software (Olympus America, Melville, NY). Exposure times and image processing conditions were identical in comparative images. Cell dimensions were then determined using the polygon length feature of Microsuite or the segmented line feature of ImageJ. Axons were measured in micrometers from their contact with the soma to the end of detectable β3 integrin staining as long as they were not branched and did not terminate on another neuron. For each condition, at least 50 lengths were determined in 3 separate trials.

4.7. Whole cell lysate preparation

Cells were harvested with trypsin-EDTA and then washed with ice-cold PBS. Pellets were immediately resuspended in ice-cold homogenization buffer, which consisted of 30 mM HEPES, pH 7.4, 2.6 mM EGTA, 20 mM MgCl₂, 80 mM β-glycerophosphate, 0.1 mM okadaic acid (Life Technologies), 0.01 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor (Sigma). Samples were then sonicated (two 5-s bursts on ice), centrifuged at 10,000×g for 15 min at 4 °C. This buffer was previously optimized for maximal CaMK-II recovery (Tombes et al., 1995, 1999). Protein concentrations were determined using the BCA assay (PIERCE, Rockford, IL).

4.8. CaMK-II activity assay

Total CaMK-II activity was assessed by measuring phosphate incorporation into a peptide substrate. Reactions were carried out in a total volume of 25 μl containing final concentrations of 20 mM HEPES (pH 7 4), 0.1 mM dithiothreitol, 15 mM magnesium acetate, 20 mM β-glycerophosphate, 0.5 μM PKA inhibitor peptide, 0.1 μM okadaic acid, 40 μM sodium orthovanadate, 0.5 mCi [γ-32P-ATP], 35 μM autocomtide-2 (peptide substrate), 1 μM calmodulin, 1 mM EGTA, and 3 mM Ca²⁺. After 10 min at 30 °C, 20 μl was pipetted onto P81 phosphocellulose paper squares that were air dried for 1 min and washed five times in 500 ml 1% phosphoric acid. Dried paper squares were quantitated by Cerenkov counting. The sequence of autocomtide-2 is KKKRQKETVDAL. These assay conditions were optimized for compatibility with the buffer in which cell lysates were prepared (Tombes et al., 1995, 1999).
4.9. Reagents

KN-93, a reversible calmodulin antagonist of CaMK-I, CaMK-II, and CaMK-IV, and KN-92, an inactive analog (Sumi et al., 1991) were obtained from Sigma Chemical Co. Other compounds were obtained from CalBiochem (La Jolla, CA). The sequence of the myristoylated auto-inhibitory peptide (myr-AIP) was KKLARGEAVDAL (BioMol, Plymouth Meeting PA). The stock concentration of these compounds was 5 mM in dH2O. For KN-KKALRRQEAVDAL (BioMol, Plymouth Meeting PA), the myristoylated auto-inhibitory peptide (myr-AIP) was used. The stock concentration was 5 mM in 20% glycerol. For KN-92 washout experiments, fresh medium was added 1 h after treatment. Neurons were imaged 2 h post-treatment. All other compounds were obtained from Sigma Chemical Co.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2006.03.099.

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