Development/Plasticity/Repair

A Novel cAMP-Dependent Pathway Activates Neuronal Integrin Function in Retinal Neurons

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Retinal neurons lose the ability to attach to and extend neurites on substrata of laminin-1 (LN-1) during late embryogenesis, in a time frame that corresponds to target innervation. Although this developmental loss correlates with a modest downregulation of integrin expression, we have shown previously that these neurons use the same laminin-binding integrins for outgrowth on other laminin isoforms to which responsivity has not been lost (Ivins et al., 1998), suggesting that integrin functional states may be a critical point of regulation. Consistent with this view, expression of an activated mutant of R-ras, an activator of integrin function, restores integrin-dependent outgrowth of late embryonic retinal neurons on LN-1 (Ivins et al., 2000). Because cyclic nucleotides have been implicated in the regulation of integrin function in non-neuronal cells, as well as in the regulation of growth cone responses to various axon growth inhibitors, we asked whether raising cAMP levels in late embryonic retinal neurons could activate neuronal integrin function and restore neurite outgrowth on LN-1. We find that, similar to R-ras expression, raising cAMP levels in these neurons promotes $\alpha 6\beta 1$ integrin-dependent neurite outgrowth. Surprisingly, these effects of cAMP are independent of protein kinase A and the EPAC (exchange protein directly activated by cAMP)/Rap pathway and suggest the existence of a novel cAMP-dependent mechanism.

Key words: **laminin; integrin; herpes viral vector; protein kinase A; EPAC; Rap1; retinal neuron; R-ras**

Introduction

The poor ability of axons to regenerate within the mammalian CNS has been attributed to many causes, from glial scars (Fitch and Silver, 1997) to molecules that inhibit axon growth (Schwab, 1996) or to intrinsic deficits in the ability of mature axons to grow (Chen et al., 1995). These possibilities involve changes in either the composition of the extracellular matrix (ECM) at the site of injury or in the ability of neurons to respond to the extracellular environment. Retinal neurons are a useful model system for studying developmentally regulated changes in neuronal responsiveness to the ECM; as embryonic development proceeds, retinal neurons lose the ability to extend neurites on the ECM protein laminin-1 (LN-1) (Cohen et al., 1986; Hall et al., 1987). Retinal neurons also lose the ability to respond to collagen IV in a similar time course (Bradshaw et al., 1995). These changes are thought to reflect a decrease in the number or activation state of integrin receptors, or both (Neugebauer and Reichardt, 1991; de Curtis and Reichardt, 1993; Ivins et al., 2000).

Extracellular ligand-binding events trigger integrin-dependent signal transduction cascades, but other signaling molecules can impinge on integrins to regulate their ability to bind ligand and signal. These include activators of protein kinase C, cAMP, the Ras-related GTPase R-ras, divalent cations, and certain conformation-sensitive anti-integrin antibodies (Hynes, 1999). However, their ability to activate integrin function depends critically on both the cellular context in which the integrins are expressed as well as the specific integrin subunits and splice variants present (Keely et al., 1999). Few studies have addressed integrin activation in the CNS (Ivins et al., 2000).

Here, we report that raising cAMP levels in CNS neurons also activates neuronal integrin function. Surprisingly, in contrast to previously reported effects of cAMP elevation in neurons, the effect of cAMP elevation on integrin activation is independent of protein kinase A (PKA). Furthermore, the effect of cAMP on integrin activation is not mimicked by cGMP nor by activation of EPAC (exchange protein directly activated by cAMP) or Rap1. These results suggest the existence of a novel cyclic nucleotide-stimulated pathway in neurons that leads to activation of neuronal integrin function.

Materials and Methods

Cell culture. Cultures of late embryonic retinal neurons were derived from embryos of timed pregnant Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), CD-1 mice (Harlan Sprague Dawley), or chicks (CBT Farms, Chestertown, MD) as described previously (Ivins et al., 1998, 2000), with the exception that tissue was enzymatically digested with papain (7.5 U/ml, 15 min, 37°C; Worthington, Freehold, NJ). Cells were plated in DMEM/F12 (1:1; Mediatech, Ormond Beach, FL) containing 0.5% BSA (crystalline; ICN Biochemicals, Costa Mesa, CA), pen/strep, glutamine, and N2 (Invitrogen, San Diego, CA) supplements. Assays of neurite outgrowth were performed in 96 well plates (3596; Costar, Cambridge, MA) at a cell density of 50,000 cells per well. Substrates were prepared as described previously (Ivins et al., 2000) using LN-1 or LN-

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2/4 (Sigma, St. Louis, MO). When present, unless stated otherwise, drugs were added to wells before the addition of cells. Cultures were incubated overnight (16–18 hr) before fixation. All experiments were repeated a minimum of three times with similar results.

Drugs and antibodies. Forskolin (FSK), isobutyl methylxanthine (IBMX), dibutyryl cAMP, 8-Bromo cGMP, H89, RpcAMPs, myristoylated PKA inhibitor protein (PKI), and SpcAMPs were from Biomol (Plymouth Meeting, PA). 8-(4-Chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate sodium salt (8CPT-2-O-Me-cAMP) was from Tocris Cookson (Ballwin, MO). The anti-β1-integrin antibody Ha2/5 and the anti-α6 antibody GoH3 were from Chemicon (Temecula, CA). Tag-1/4D7 and RT97-secreting hybridomas were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and used as undiluted supernatants to stain cultures. Rabbit anti-Gap43 (Abcam, Cambridge, MA) was used at 1:500. Rabbit anti-neurofilament H (Chemicon) was used at 1:1000. The anti-myc-secreting hybridoma 9E10 was from American Type Culture Collection (Manassas, VA) and used as an undiluted supernatant to stain cultures. The anti-HA monoclonal Ha.11 (Covance, Princeton, NJ) was used at 1:1000. All second antibodies were alexa-fluor conjugates (Molecular Probes, Eugene, OR).

Analysis. After antibody staining, cultures were examined on a Nikon (Tokyo, Japan) TE2000U microscope. Images were captured using a CoolSnap ES CCD camera (Roper Scientific, Tucson, AZ) and MetaMorph software (Universal Imaging Corporation, West Chester, PA). Statistical analysis was performed using SigmaStat.

PKA assays. The MESACUP protein kinase assay kit (MBL, Nagoya, Japan) was used to assay activation of PKA according to the protocol of the manufacturer. Acutely dissociated chick neural retina cells were incubated in suspension in complete media with drugs as indicated for 30 min at 37°C. After incubation, cells were rinsed with fresh media, collected by centrifugation, lysed in ice-cold sample preparation buffer, and centrifuged at 14,000 rpm in a microfuge. Supernatants were incubated in buffer containing ATP and added to a PKA pseudosubstrate-coated microplate. After incubation, reactions were stopped, and wells were incubated with a biotinylated antibody that recognizes the PKA-phosphorylated form of the PKA pseudosubstrate adsorbed on the microwells. Wells were then incubated with alkaline peroxidase-conjugated streptavidin, followed by substrate solution. The reaction was stopped, and the optical density of each sample was determined at 492 nm using a microwell plate reader. Determinations were made in triplicate.

cDNA construction, viral packaging. cDNAs encoding wild-type human Rap1A and Rap1B were obtained from the Guthrie cDNA resource center (Sayre, PA). Using standard PCR mutagenesis, each cDNA was modified to encode the HA epitope tag at the N terminus. Activating mutations (G12V) were also created. After confirmation of cDNA sequences by direct sequencing, cDNAs were subcloned into the herpes simplex virus (HSV) amplicon pHSV-Ires-green fluorescent protein and packaged into HSV virions as described previously (Ivins et al., 2000). Cultures were infected within 1 hr of cell plating at a multiplicity of infection of 0.5–1.0. The preparation of R-ras-expressing HSV amplicons was described previously (Ivins et al., 2000).

Results

Recent studies have implicated changes in neuronal cyclic nucleotide levels in regulation of neuronal responses to a variety of axon guidance cues (Ming et al., 1997; Song et al., 1997; Chalasani et al., 2003; Guirland et al., 2003). Raising cAMP levels, either pharmacologically or through activation of G-protein-coupled receptors, has also been shown to activate integrin function in a number of non-neuronal cell types (Enserink et al., 2002) through activation of a cyclic nucleotide-activated guanine nucleotide exchange factor, EPAC, and its effector protein, Ras-related GTPase Rap1. We sought to determine whether cAMP levels regulate neuronal integrin function and, if so, whether such regulation is mediated through PKA, the EPAC/Rap1 pathway, or a novel mechanism.

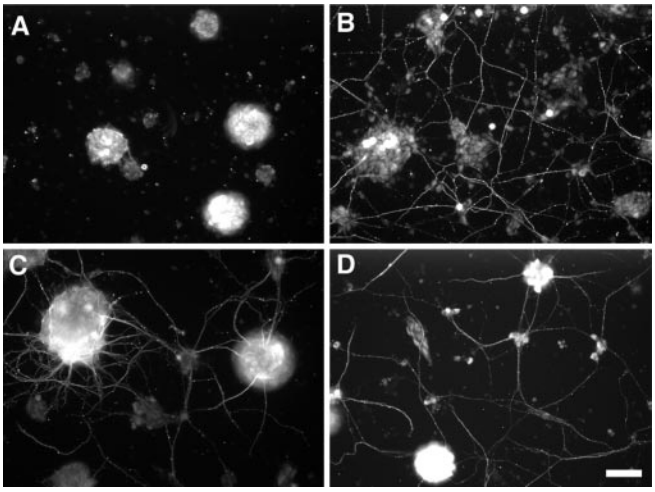

Figure 1. Effect of PKA inhibition on cAMP-dependent neurite outgrowth. Dissociated retinal neurons from E18 rats were plated in serum-free media on LN-1 or LN-2 in the presence or absence of FSK/IBMX, allowed to grow overnight, and were then fixed, stained for neurofilament with mAb RT97, and photographed. *A*, *B*, Control cultures on LN-1 (*A*) and LN-2 (*B*) are shown. Cultures in *C* and *D* were plated on LN-1 in the presence of FSK/IBMX; the culture in *D* was further treated with a mixture of PKA inhibitors (H89, 4 μM; PKI, 4 μM; RpcAMPS, 500 μM). Scale bar (in *D*), 50 μm.

Raising cAMP levels activates β1 integrin function and restores neurite outgrowth on LN-1

Treatment of cultures of late embryonic rat retinal neurons grown on LN-1 substrata with dibutyryl cAMP (0.5–2.0 mM; data not shown) or with a combination of forskolin (5 μM) and the phosphodiesterase inhibitor IBMX (100 μM), denoted FSK/IBMX, restores neurite outgrowth on LN-1 (Fig. 1). At these concentrations, FSK and IBMX alone do not promote outgrowth but, in combination, promote outgrowth to levels comparable with that seen on LN-2. To confirm that some of the responding cells were retinal ganglion cells (RGCs), we stained cultures for the RGC marker TAG-1. A number of neurite-bearing cells was found to express both neurofilament and TAG-1 (data not shown).

In initial experiments with both rat and mouse retinal cultures, the function-blocking anti-β1 integrin antibody Ha2/5 (10 μg/ml) reduced neurite growth in FSK/IBMX-treated cultures to levels indistinguishable from control, untreated cultures on LN-1, confirming the β1-integrin dependence of the phenomenon. Similar results were obtained in embryonic day (E) 10 chick retinal neuronal cultures using the function-blocking anti-β1 monoclonal antibody (mAb) W1B10, confirming that this effect is not species specific. Treatment of E16 mouse retinal cultures with the function-blocking mouse-specific rat anti-α6 integrin antibody GoH3 (5 μg/ml) also completely blocked the stimulation of outgrowth by FSK/IBMX (LN-1: 4.9 $\pm$ 14.3 μm, $n = 66$ neurites; LN-1+FSK/IBMX: 46.0 $\pm$ 52.0 μm, $n = 67$ neurites; LN-1+FSK/IBMX+GoH3: 3.3 $\pm$ 10.5 μm, $n = 49$ neurites; LN-1+FSK/IBMX+Ha2/5: 9.9 $\pm$ 22.9 μm, $n = 57$ neurites; values are mean lengths $\pm$ SD). This suggests that, as previously reported for R-ras-stimulated integrin activation and neurite outgrowth (Ivins et al., 2000), these effects of cyclic nucleotides are mediated by activation of the $\alpha 6\beta$1-integrin.

To confirm that these effects were specifically attributable to cAMP, several additional experiments were performed. Because IBMX is a nonselective phosphodiesterase inhibitor and may therefore also affect cGMP levels, we treated cultures of E18 rat

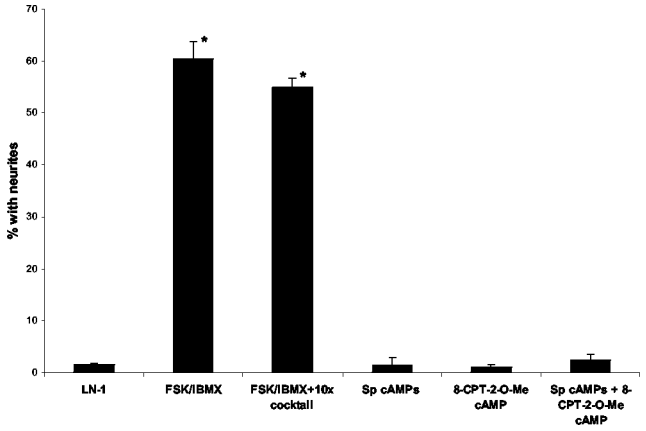

Figure 2. Effects of selective PKA and EPAC activators on neurite outgrowth. E18 rat retinal neurons plated on LN-1 substrata in the presence of drugs as indicated were cultured overnight and then fixed and stained for neurofilament with RT97. The percentage of cells or cell clumps that bore neurites more than two cell body diameters in length is shown. Even at high concentrations, PKA inhibitors did not inhibit FSK/IBMX-stimulated neurite outgrowth on LN-1 (overall significance by one-way ANOVA; $p < 0.001$; Holm–Sidak *post hoc* analysis; LN-1 ± SpcAMPs are equivalent; the mixture of inhibitors had no effect on FSK/IBMX-stimulated outgrowth). Neither SpcAMPs (100 μM), 8CPT-2Me-cAMP (100 μM), nor a combination of these drugs (100 μM each) stimulated neurite outgrowth on LN-1 above control levels (overall significance by one-way ANOVA; $p < 0.001$; Holm–Sidak *post hoc* analysis; only FSK/IBMX stimulates outgrowth above control levels).

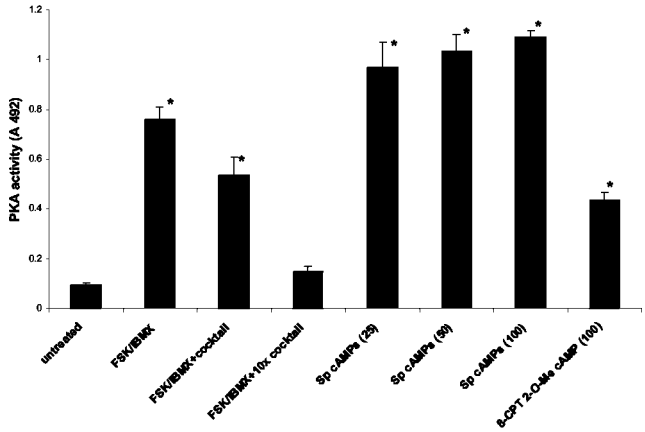

Figure 3. PKA activity in FSK/IBMX-stimulated retinal neurons. Acutely dissociated E10 chick retinal neurons were treated in suspension with drugs as indicated for 30 min, and cell lysates were assayed for PKA activity. The inhibitor mixture consisted of H89 (400 nM or 4 μM), PKI (400 nM or 4 μM), and RpcAMPs (50 μM or 500 μM). FSK/IBMX and FSK/IBMX + 1× mixture are significantly different than control. FSK/IBMX + 10× mixture and control are indistinguishable. SpcAMPs (25–100 μM) stimulated PKA to levels equivalent to or greater than FSK/IBMX. The EPAC-selective activator 8-CPT 2-O-Me cAMP (100 μM) also stimulated PKA activity above control levels (overall significance by one-way ANOVA; $p < 0.01$; Holm–Sidak *post hoc* test; *, groups different than control).

retina plated on LN-1 with 8-Bromo-cGMP (0.5–2.0 mM; data not shown). These treatments were without effect. In addition, we treated cultures plated either on LN-1 in the presence or absence of FSK/IBMX or on LN-2, with the adenylate cyclase (AC) inhibitor SQ22536 (50 μM). Treatment with SQ22536 neither stimulated neurons plated on LN-1 nor inhibited neurite outgrowth on LN-2 but completely inhibited FSK/IBMX-stimulated outgrowth on LN-1 (data not shown). This confirms an essential role of AC in FSK/IBMX-stimulated neurite outgrowth on LN-1 and also suggests that basal levels of AC activity are not required to support β1 integrin-dependent neurite growth on LN-2.

Effects of cAMP on neuronal integrin function are independent of protein kinase A activity

To determine whether the effects of raising neuronal cAMP on neurite outgrowth were attributable to activation of PKA, we treated cultures with a battery of pharmacological blockers of PKA (Figs. 1*D*, 2). Surprisingly, none of the inhibitors tested affected cAMP-dependent neurite outgrowth on LN-1, nor did they inhibit outgrowth on LN-2 (data not shown). Because cAMP activates the same $\alpha 6\beta 1$-integrin that is activated by R-ras expression, we also examined the effects of inhibition of PKA on R-ras^{G38V}-stimulated outgrowth on LN-1. These compounds also did not affect R-ras^{G38V}-stimulated outgrowth on LN-1 (data not shown).

Because of concerns over inhibitor specificity when used at high concentrations, we prepared a mixture of PKA inhibitors containing H89, RpcAMPs, and PKI (H89, 400 nM or 4 μM; RpcAMPs, 50 μM or 500 μM; PKI, 400 nm or 4 μM, referred to as 1× or 10×, respectively) and applied it to cultures of late embryonic retinal neurons (Figs. 1, 2). Even the 10× mixture did not inhibit FSK/IBMX-stimulated neurite outgrowth on LN-1 (Figs. 1, 2). Furthermore, the 10× mixture did not inhibit outgrowth on LN-2 or outgrowth stimulated by R-ras^{G38V} expression (data not shown). Identical results were obtained when cultures were pretreated with inhibitors for 1 hr before the introduction of FSK/IBMX (data not shown). Because these compounds inhibit PKA through three distinct mechanisms and were used at concentrations well exceeding the IC_{50} for PKA, these results suggest that the effects of cAMP on neuronal integrin function occur independently of PKA activity.

To confirm the effectiveness of this PKA-blocking mixture, we measured PKA activity directly by ELISA (Fig. 3). As expected, FSK/IBMX treatment of retinal neurons results in a large increase in PKA activity above basal levels (sevenfold to eightfold). Although treatment with the 1× mixture of H89, RpcAMPs, and PKI inhibited PKA activity somewhat, treatment with the 10× mixture reduced PKA activity to control levels.

To test whether PKA activity was sufficient to promote integrin-dependent neurite outgrowth, we treated cultures with the selective PKA activator SpcAMPs (Christensen et al., 2003). SpcAMPs (25–100 μM) did not stimulate neurite outgrowth from late embryonic retinal neurons on LN-1 (Fig. 2). Few neurites were present on LN-1 in cultures of E18 rat retina treated with or without 100 μM SpcAMPs (Fig. 2), and no differences in neurite lengths were observed (LN-1: 137.8 ± 81.1 μm, $n = 43$ neurites; LN-1+SpcAMPs: 137.3 ± 91.6 μm, $n = 51$ neurites; values are means ± SD). Measurements of PKA activity in extracts of cells treated with varying concentrations of SpcAMPs confirmed that SpcAMPs was indeed activating PKA (Fig. 3). Together, these results demonstrate that PKA activation is neither necessary nor sufficient for cAMP-stimulated integrin-dependent neurite growth of late embryonic retinal neurons on LN-1.

Effects of cAMP on neuronal integrin function are independent of EPAC and Rap1

EPAC, a guanine nucleotide exchange factor for Rap1, is activated by cAMP through the binding of cAMP to a cAMP-binding regulatory domain (Enserink et al., 2002) and provides an alternate, PKA-independent pathway leading from cAMP to integrin activation (Bos et al., 2003; Rangarajan et al., 2003). A potential role of EPAC and Rap1 signaling in cAMP-stimulated neurite outgrowth of retinal neurons was tested in two experiments. First, we treated cultures of E18 rat retinal neurons with an

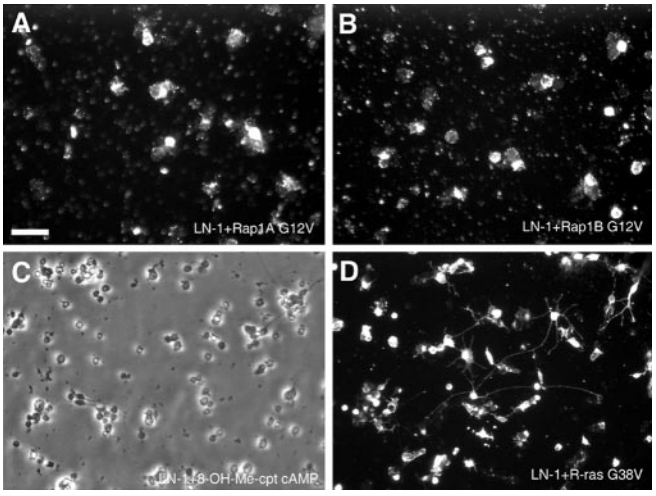

Figure 4. Effect of EPAC/Rap pathway activation on neurite outgrowth. *A–D*, E18 rat retinal neurons were plated on LN-1 and infected with HSV amplicons driving expression of Rap1A^{G12V} (*A*), Rap1B^{G12V} (*B*), or R-ras^{G38V} (*D*) or treated with the selective EPAC activator 8CPT-2-O-Me-cAMP (*C*). Only R-ras^{G38V} expression promotes outgrowth on LN-1. Scale bar (in *A*), 50 μm.

EPAC-selective cAMP analog, 8CPT-2-O-Me-cAMP (Christensen et al., 2003) at concentrations ranging from 1 to 100 μM (EC_{50}, 2.2 μM for EPAC1) (Enserink et al., 2002). Even at the highest concentrations tested, no stimulation of neurite outgrowth on LN-1 was observed (Figs. 2, 4). Second, we prepared HSV amplicon vectors to drive expression of constitutively activated mutants of Rap1A (Rap1A^{G12V}) and Rap1B (Rap1B^{G12V}) and infected cultures of E18 rat retinal neurons (Fig. 4). Neither construct stimulated neurite outgrowth above background levels seen in control cells on LN-1. We also prepared HSV amplicons driving expression of wild-type Rap1A and Rap1B and used these to infect cultures of E18 rat retinal neurons. Not only did Rap1 expression have no stimulatory effect, but concurrent treatment with either SpcAMPs (100 μM) to activate PKA or 8CPT-2-O-Me-cAMP (100 μM) to activate EPAC was also without effect (data not shown). We also treated cultures with both SpcAMPs and 8CPT-2-O-Me-cAMP (25–100 μM each) and quantified neurite outgrowth on LN-1 (Fig. 2). No activation was seen, although at these concentrations, 8CPT-2-O-Me-cAMP also stimulates PKA activity close to levels seen with FSK/IBMX (Fig. 3). Finally, we simultaneously treated cultures with SpcAMPs, 8CPT-2-O-Me-cAMP, and 8-Bromo-cGMP. This combination did not stimulate outgrowth on LN-1 (data not shown). From these experiments, we conclude that the cAMP-stimulated integrin-dependent neurite outgrowth that we observed in late embryonic retinal neurons is independent of both PKA and the EPAC/Rap pathway and must therefore be mediated by a novel cAMP-dependent pathway.

Discussion

Late embryonic retinal neurons have been a useful model system to study the developmental loss of integrin function that occurs in many populations of CNS neurons (Cohen et al., 1986; Neugebauer and Reichardt, 1991; de Curtis and Reichardt, 1993; Ivins et al., 2000). Previously, we found that either Mn++ treatment or expression of a constitutively activated mutant of the Ras-related GTPase R-ras activates integrin function and restores integrin-dependent neurite outgrowth on substrata of LN-1 (Ivins et al., 2000). cAMP levels have also been shown to decline developmentally in several neuronal types (Cai et al., 2001; Shewan et al., 2002) and, importantly, raising cAMP levels improves the regenerative capability of sensory neurons *in vitro* and *in vivo* (Neumann et al., 2002; Qiu et al., 2002). Because cAMP levels have been shown to regulate integrin functional states in several non-neuronal cells (Rangarajan et al., 2003), we asked whether raising cAMP levels in late embryonic retinal neurons would restore integrin-dependent neurite outgrowth on LN-1. We found that, like treatment with Mn++ or R-ras^{G38V}, raising neuronal cAMP levels does restore integrin-dependent neurite outgrowth on LN-1. However, this activity is independent of PKA activation, EPAC/Rap1 signaling, and activation of cyclic nucleotide-gated calcium channels, suggesting that it occurs by a novel and as-yet undescribed cAMP-dependent mechanism.

Modulation of neuronal cAMP levels has been shown to alter growth cone behavior toward a number of axon guidance cues. These cues include BDNF (Song et al., 1997), semaphorins (Chalasani et al., 2003), netrin-1 (Shewan et al., 2002), and myelin-associated glycoprotein (Cai et al., 2001; Qiu et al., 2002). In every case, a strict PKA dependence was observed. For the most part, however, these studies were not performed under conditions in which the contributions of integrin signaling could be evaluated. Toward this end, it is noteworthy that laminin-induced signaling can convert the attraction of *Xenopus* spinal neurons toward a netrin-1 gradient into repulsion (Hopker et al., 1999). Similarly, the response of *Xenopus* retinal ganglion cell growth cones to ephrin A5 has been reported to be dependent on the growth substratum (Weinl et al., 2003). In the future, it will be of great interest to determine whether the promotion of integrin signaling can contribute to the abrogation of responses to axon growth inhibitors.

Integrins and integrin signaling has been implicated in a wide range of cellular events. These include growth factor signaling (Colognato et al., 2002), apoptosis (Frisch and Ruoslahti, 1997), neurite growth and growth cone guidance (Georges-Labouesse et al., 1998), and signaling by Ig-superfamily members (Treubert and Brummendorf, 1998; Lilien et al., 1999). The mechanisms through which integrins participate in such a wide variety of signaling events are unclear. One possibility is that post-translational modifications, such as palmitoylation, allow or promote the interactions of multiple signaling proteins within cell-surface microdomains (Yang et al., 2002). Consistent with this, integrin activation by R-ras in non-neuronal cells requires not only GTP to be bound, but also palmitoylation to occur at a site near the R-ras *c* terminus (Oertli et al., 2000).

In PC12 cells, activation of both PKA and EPAC is necessary for cAMP to promote neurite outgrowth (Christensen et al., 2003), and both activities synergize with NGF to promote differentiation. Whether similar pathways operate in primary neurons, either central or peripheral, has yet to be determined. However, our present results would suggest that distinct or additional pathways operate in neurons. To date, the ability of the EPAC/Rap pathway to activate integrin function has only been investigated in non-neuronal cells (Bos et al., 2003; Rangarajan et al., 2003). Our data with 8CPT-2-O-Me-cAMP and forced Rap1 expression suggests that activation of the Rap pathway alone is not sufficient to promote activation of laminin-binding integrins in retinal neurons, nor is it sufficient when activated in conjunction with PKA.

The mechanism by which R-ras activates integrin function is currently unknown. Point mutations in the effector loop can selectively inhibit the ability of R-ras to interact with its effectors, Raf-1, PI-3 kinase, nore1, and RalGDS (Oertli et al., 2000). However, inhibition of these interactions has not been found to correlate with the ability of R-ras to activate integrin function, sug-

gesting that additional, unidentified effectors may be responsible (Oertli et al., 2000). The mechanism through which cAMP affects neuronal integrins is likewise unknown, and the present data suggest that it is independent of all currently known pathways through which cAMP signals. It is highly likely that retinal neurons express a cAMP-binding protein of which the cAMP-binding domain is dissimilar to those of PKA and EPAC.

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