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Influence of cAMP and protein kinase A on neurite length from spiral ganglion neurons

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Abstract

Regrowth of peripheral spiral ganglion neuron (SGN) fibers is a primary objective in efforts to improve cochlear implant outcomes and to potentially reinnervate regenerated hair cells. Cyclic adenosine monophosphate (cAMP) regulates neurite growth and guidance via activation of protein kinase A (PKA) and *Exchange Protein directly Activated by Cyclic AMP* (Epac). Here we explored the effects of cAMP signaling on SGN neurite length *in vitro*. We find that the cAMP analog, cpt-cAMP, exerts a biphasic effect on neurite length; increasing length at lower concentrations and reducing length at higher concentrations. This biphasic response occurs in cultures plated on laminin, fibronectin, or tenascin C suggesting that it is not substrate dependent. cpt-cAMP also reduces SGN neurite branching. The Epac-specific agonist, 8-pCPT-2'-O-Me-cAMP, does not alter SGN neurite length. Constitutively active PKA isoforms strongly inhibit SGN neurite length similar to higher levels of cAMP. Chronic membrane depolarization activates PKA in SGNs and also inhibits SGN neurite length. However, inhibition of PKA fails to rescue neurite length in depolarized cultures implying that activation of PKA is not necessary for the inhibition of SGN neurite length by chronic depolarization. Expression of constitutively active phosphatidylinositol 3-kinase, but not c-Jun N-terminal kinase, isoforms partially rescues SGN neurite length in the presence of activated PKA. Taken together, these results suggest that activation of cAMP/PKA represents a potential strategy to enhance SGN fiber elongation following deafness; however such therapies will likely require careful titration so as to simultaneously promote rather than inhibit nerve fiber regeneration.

Keywords

protein kinase A; axon; cyclic adenosine monophosphate; phosphatidylinositol 3-kinase; c-Jun N-terminal kinase

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INTRODUCTION

Spiral ganglion neurons (SGNs) are bipolar neurons that provide the afferent fibers of the auditory nerve relaying information from the cochlea to the cochlear nucleus in the brainstem. The peripheral fibers of SGNs, sometimes referred to as dendrites, innervate hair cells in the organ of Corti and the central fibers terminate in the cochlear nucleus. Typical of axons, both the central and peripheral branches of SGNs are myelinated, conduct action potentials, and express axonal microtubules and here will be referred to as central and peripheral fibers, respectively.

SGNs are dependent on neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), for their development and continued survival (Fritzsch et al., 1997; Hegarty et al., 1997; Lefebvre et al., 1994; Malgrange et al., 1996; McGuinness et al., 2005; Miller et al., 1997; Mou et al., 1997; Staecker et al., 1996). Chronic membrane depolarization with elevated extracellular potassium also promotes SGN survival *in vitro* by activating multiple intracellular signaling cascades including protein kinase A (PKA), Ca^{2+} /calmodulin-dependent protein kinase (CaMK) II, and CaMKIV (Bok et al., 2003; Bok et al., 2007; Hansen et al., 2001; Hansen et al., 2003; Hegarty et al., 1997).

Electrical stimulation has also been shown to enhance SGN survival *in vivo* in some studies (Leake et al., 1999; Miller et al., 2003), but not others (Agterberg et al., 2010; Shepherd et al., 1994). Deafness caused by loss of hair cells due to noise trauma, ototoxic drugs, aging, or genetic disorders results in gradual degeneration of the SGN peripheral fibers innervating the organ of Corti and eventually loss of the neurons (Alam et al., 2007; Altschuler et al., 1999; Bao et al., 2010; Dodson et al., 2000; Lee et al., 2003; Spoendlin, 1975; White et al., 2000). In humans, SGN loss following deafening occurs much more slowly allowing for the functional stimulation of the auditory nerve with implanted electrodes (Gomaa et al., 2003; Khan et al., 2005; Linthicum et al., 2009). Nevertheless, most human cochleae with extensive hair cell loss have significantly fewer peripheral fibers and reduced SGN numbers (Nadol, 1990; Zimmermann et al., 1995). So far there is no clear evidence that electrical stimulation via cochlear implantation enhances SGN survival in humans (Khan et al., 2005; Linthicum et al., 1991; Nadol et al., 2001).

Cochlear implants provide significant speech understanding to deafened patients yet the implant poorly represents complex auditory stimuli such as speech in background noise and music. Induction of SGN peripheral fiber growth to be in close proximity or even contact the stimulating electrodes should allow for lower activation thresholds and, perhaps, improved functional outcomes (Goldwyn et al., 2010; Rubinstein, 2004; Xu et al., 2008). Development of methods to maintain or regenerate SGNs and their peripheral fibers following deafness represents a primary objective to improve the functional outcomes achieved with cochlear implants (Bianchi et al., 2004; Gillespie et al., 2005; Pettingill et al., 2007; Richardson et al., 2009; Roehm et al., 2005; Staecker et al., 2010). Regeneration of peripheral SGN fibers to innervate new hair cells will also be required if strategies aimed at restoring hearing by replacing lost hair cells with stem cells or genetically modified supporting cells becomes feasible in the future. Thus, there is lively interest in understanding the signaling events that influence SGN neural regeneration.

Neuron cultures, including SGNs, facilitate manipulation of the intra- and extracellular environments and these have been leveraged to dissect the complex molecular events regulating nerve fiber growth. In culture, SGNs extend long processes that will be referred to here as neurites. These neurites contain axonal microtubules and terminate in growth cones (Atkinson et al., 2011; Li et al., 2010). Most cultured SGNs extend a single neurite, although bipolar SGNs are also evident in culture (Whitlon et al., 2006). Treatment of spiral

ganglion cultures with cytokines such leukemia inhibitory factor increases the percentage of surviving SGNs that extend one or more neurites (Whitlon et al., 2006). Although neurite growth *in vitro* does not completely recapitulate nerve fiber regeneration *in vivo*, primary neuronal cultures provide a model that facilitates the manipulation of signaling events at multiple levels and the characterization of fundamental activities that govern neural survival and regeneration.

Cyclic adenosine monophosphate (cAMP) is a key intracellular signal that regulates neuronal differentiation, survival, neurite length, and neurite guidance in a variety of neurons including mammalian sensory and central nervous system neurons (Aglah et al., 2008; Cai et al., 2001; Fujioka et al., 2004; Hanson et al., 1998; Hegarty et al., 1997; Monastersky et al., 1984; Neumann et al., 2002; Song et al., 1997). cAMP exerts its effects on neurites and growth cones through activation of protein kinase A (PKA) and the Rap1 guanine-nucleotide exchange factor protein, *Exchange Protein directly Activated by Cyclic AMP* (Epac) (Aglah et al., 2008; Christensen et al., 2003; Han et al., 2007; Kopperud et al., 2003; Murray et al., 2008; Murray et al., 2009). Treatment of cultured SGNs with cell permeant cAMP analogs promotes survival and facilitates neurite growth in the presence of inhibitory central glia indicating that SGNs respond to cAMP signaling (Hansen et al., 2001; Hegarty et al., 1997; Jeon et al., 2011). Here we sought to determine the influence of cAMP and PKA signaling on neurite length from cultured neonatal rat SGNs. We found that cAMP exerts a biphasic effect on neurite length with high concentrations reducing length, an effect that appears to be mediated by PKA activity and is independent of culture substrate. Expression of phosphatidylinositol 3-kinase (PI3-K), but not c-Jun N-terminal kinase (JNK), constitutively active isoforms rescued neurite length in SGNs with activated PKA.

METHODS

Spiral ganglion culture

Dissociated spiral ganglion cultures were prepared as previously described (Hansen et al., 2001; Hegarty et al., 1997). Briefly, spiral ganglion neurons were collected from P5 rat pups and incubated in 0.25% trypsin/EDTA and 0.2% collagenase in Hank's buffered salt solution at 37°C for 15–20 min. SGNs were then dissociated in Dulbecco's modified Eagle's medium supplemented with N₂ (Invitrogen, Carlsbad, CA) and fresh insulin (Sigma, St. Louis, MO; 10 µg/ml) and plated on polyornithine/mouse laminin (Sigma, 20 µg/ml) coated eight well plates (Nalge Nunc, Naperville, IL). Subsets of cultures were plated on eight well plates coated with human fibronectin (Sigma, 20 µg/ml) or human tenascin C (Sigma, 20 µg/ml). Cultures were maintained at 37°C in a humidified incubator with 6.0% CO₂. NT-3-positive control cultures were grown in NT-3 (R&D Systems, Minneapolis, MN, 50 ng/ml) whereas NT-3-negative control cultures lacked exogenous NT-3. Subsets of cultures were treated with cpt-cAMP, forskolin (FSK), 8-pCPT-2'-O-Me-cAMP (200 µM) or Rp-cAMPS (1 mM) (all from EMD Chemicals, Gibbstown, NJ). All biochemicals were dissolved in culture medium except FSK, which was dissolved in dimethyl sulfoxide (DMSO, 0.08%). Control cultures for the FSK-treated experiments included DMSO. Chronic membrane depolarization was accomplished by elevating extracellular K⁺ to 30 mM or 80 mM, as previously described (Hansen et al., 2001; Hegarty et al., 1997; Roehm et al., 2008).

Spiral ganglion neuron gene transfer

Six to eight hours after plating, dissociated SG cultures were transfected with expression plasmids using calcium phosphate precipitation as previously described (Hansen et al., 2007; Hansen et al., 2003; Zha et al., 2001). GPKA, GPKAnls, GPKAnes, GPKI, expression plasmids were kindly provided by Dr. Steven Green (University of Iowa, Iowa City, IA) (Bok et al., 2003). Green fluorescent protein (GFP), MKK-JNK1, and p110 expression

plasmids have previously been described (Atkinson et al., 2011; Hansen et al., 2007; Renton et al., 2010). For co-transfections the ratio of plasmids was 1:1. Twelve hours after transfection, the medium was exchanged with NT-3 culture medium for 48 h unless indicated. Cultures were then fixed and immunolabeled. The transfection efficiency for rat neonatal SGNs using this protocol is ~10% as previously reported (Bok et al., 2003; Hansen et al., 2007; Hansen et al., 2003; Zha et al., 2001). Approximately 5–10% of non-neuronal cells are also transfected.

Immunolabeling and microscopy

Cultures were fixed with 4% paraformaldehyde for 10 min. and permeabilized with 0.1% Triton-X in phosphate-buffered saline (PBS) for 15 min. Blocking buffer, comprised of 5% goat serum, 2% bovine serum albumin (Sigma), and 0.1% Triton-X in PBS, was added to each well for 20 min. Cultures were then immunolabeled with an anti-neurofilament 200 (NF200) monoclonal antibody (Sigma, #N52, 1:1000) that recognizes phosphorylated and unphosphorylated NF200. Alexa 568-conjugated secondary antibodies (Invitrogen, 1:800) were used to detect NF200 immunolabeling. Digital images were captured with a Leica DMR III microscope equipped with epifluorescent filters and a cooled CCD camera using Leica FW4000 software (Leica Microsystems, Bannockburn, IL).

GFP fluorescence was quantified from digitized images using ImageJ software (NIH, Bethesda, MD, <http://rsb.info.nih.gov/ij/>) as previously described (Roehm et al., 2008). A region of interest outline ~20 μ m in diameter was drawn on each transfected (GFP-positive) neuron (NF200-positive) and the mean pixel density of the selection recorded. For each image, the background fluorescence was subtracted by measuring the mean pixel density of an adjacent non-transfected cell. Images of each SGN were captured using the same exposure time and light intensity and at the same sitting. To prevent photobleaching, SGNs were not imaged or exposed to light prior to capturing the image used for quantification of GFP fluorescence. To ensure that the images would not be saturated, the exposure time was initially established based on the histogram of a bright cell and none of the images exhibited a pixel intensity of >230 (0–255 scale), indicating that each was not saturated.

Determination of neurite length and branching and of neuron survival

Neurite length was determined as previously described from digital images of 7–10 randomly selected 10 $\times$ or 20 $\times$ fields, with roughly equal cell density, for each experimental condition (Atkinson et al., 2011; Hansen et al., 2007; Roehm et al., 2008). Neurite length was determined by measuring the entire length of the longest process from each neuron using the measurement tool in ImageJ. Length was defined as the maximal possible distance along a neurite, i.e., the distance from the soma to the end of the longest neurite (if more than one neurite was present) and to the end of the longest branch at each branch point for branched neurites. If the longest neurite extended beyond the image border, additional images were acquired to include and measure the entire length of the process (Hansen et al., 2007; Roehm et al., 2008). Each condition was performed in duplicate and repeated with at least 3 separate culture preparations; thus at least 6 wells were scored for each condition. At least 60 neurites (~30/well) were measured for each repetition. For transfected SGNs each condition was also performed in duplicate and repeated with at least 3 separate culture preparations. Only GFP-positive neurons were imaged and analyzed. Images were captured of the first ~15 transfected neurons encountered as the observer scanned through each well from top to bottom. The mean neurite length for each experimental repetition and standard error of the mean (SEM) was determined using Excel software (Microsoft, Seattle, WA) (Atkinson et al., 2011; Renton et al., 2010). Significance for differences in neurite length among treatment groups was determined by a one-way ANOVA followed by a Holm-Sidak post-hoc comparison in SigmaStat (Systat Software, Chicago, IL).

Neurite branching was determined by scoring total number of primary, secondary, tertiary, and quaternary neurites was determined for each neuron in a subset of cultures. Since longer neurites would have great opportunity for branching along their course, the total number of neurite processes was then divided by the length of the longest neurite to establish the branching index. The primary branching index included all neurites while the secondary branching index excluded the primary neurites considering secondary, tertiary and quaternary neurites. In a subset of cultures, we also determined the percent of SGNs that failed to grow a neurite by dividing the number of SGNs that failed to extend a neurite (defined as a NF200-positive process $<10\ \mu\text{m}$) by the total number of scored SGNs.

Neuron survival was determined as previously described by counting the number of SGN 10 randomly selected 20X fields in each well (Jeon et al., 2011). Experiments were performed in duplicate and repeated with at least 3 separate culture preparations; thus at least 6 total wells were scored for each condition. Criteria for a surviving neuron included NF200-positive with an intact, non-pyknotic nucleus. Due to the variability in neuron numbers that are plated in each experiment, survival in each condition was normalized and expressed as a percent of the survival of SGNs maintained in NT-3. For transfected SGNs, only GFP-positive, NF200-positive neurons were scored. The number of GFP-positive surviving SGNs in NT-3 across all repetitions was 98 ± 15 (mean $\pm$ standard deviation). Significance for differences in SGN survival among treatment groups was determined by a one-way ANOVA followed by a Holm-Sidak post-hoc comparison in SigmaStat (Systat Software, Chicago, IL).

RESULTS

The cell-permeant cAMP analog, cpt-cAMP, exerted a biphasic effect on neurite length and reduced neurite branching

To begin to explore the consequences of cAMP signaling on SGN neurite length, we treated dissociated SG cultures with cpt-cAMP (0.1–10 mM), a cell-permeant cAMP analog. To ensure SGN survival and promote neurite growth, cultures were maintained in NT-3 unless otherwise noted. SGNs displayed a biphasic response to elevated cAMP levels; 1 mM significantly increased neurite length ($p<0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison) whereas 10 mM significantly reduced neurite length ($p<0.05$) compared with NT-3 alone (Fig. 1). In the absence of NT-3, 1 mM cpt-cAMP was significantly less effective at increasing neurite length compared to NT-3 ($p<0.05$), even though cpt-cAMP at this concentration promotes SGN survival (Fig. 1), as previously shown (Hansen et al., 2001; Hegarty et al., 1997). In 10 mM cpt-cAMP, 21.91% of SGNs failed to develop a neurite (Supplemental Table I) and neurite length was significantly less than NT-3-positive control cultures; however, a small fraction of SGNs (~5%) developed extensive fine neurites yet failed to form a dominant neurite typical of SGNs in control conditions (Fig. 1D). SGN survival in cultures maintained in 10 mM cpt-cAMP and NT-3 was comparable to survival in cultures maintained in NT-3 alone (Fig. 1, $p>0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison) suggesting that the higher level of cpt-cAMP was not generally toxic to the SGNs. Further, SGNs maintained in 10 mM cpt-cAMP displayed healthy non-pyknotic nuclei (Supplemental Fig. 1).

We also measured SGN neurite length in cultures treated with forskolin (FSK, 0.1–10 μM), an adenylate cyclase activator. In this case, cultures treated with FSK demonstrated significantly longer neurites compared with NT-3-positive control cultures ($p<0.05$, each condition). In contrast to cpt-cAMP, FSK increased neurite length at all concentrations; even higher doses did not reduce neurite length. Supplemental Table I presents the percent of SGNs in each condition for Figures 1 and 4 that did not extend a neurite.

We also determined the influence of cAMP signaling on SGN neurite branching. Treatment with 10 mM cpt-cAMP significantly increased the primary branching index compared to cultures in NT-3 (Fig. 2). However treatment with cpt-cAMP reduced the secondary branching index in a dose dependent manner compared with cultures in NT-3 ($p < 0.05$ each, one way ANOVA followed by a Holm-Sidak post-hoc comparison). The increased primary branching index and reduced secondary branching index in cultures treated with 10 mM cpt-cAMP indicates that at this high concentration, cpt-cAMP increased the number of primary processes that extend from some of the SGNs although these small, primary processes do not branch significantly nor do they form dominant processes comparable to SGNs in NT-3.

The biphasic effect of cpt-cAMP on SGN neurite length was independent of substrate

Elevation of cAMP promotes or inhibits embryonic chick DRG neurite growth depending on the substrate on which the neurons are plated (Lemons et al., 2006). We considered the possibility that the effect of cpt-cAMP on SGN neurite length was likewise substrate-dependent. SGNs were plated on fibronectin or tenascin C and treated with cpt-cAMP. The biphasic effect of cpt-cAMP on SGN neurite length persisted on both substrates (Fig. 3); 1 mM cpt-cAMP significantly increased neurite length while 10 mM cpt-cAMP significantly reduced neurite length ($p < 0.05$, each), similar to the response in cultures plated on laminin. Thus, the biphasic response of SGN neurites to cpt-cAMP does not appear to be substrate dependent.

The EPAC agonist, 8-pCPT-2'-O-Me-cAMP, did not alter SGN neurite length

Elevation of cAMP activates both PKA and Epac, a Rap1 guanine-nucleotide exchange factor, and, depending on the developmental stage and extent of cAMP elevation, both have been implicated in regulating neurite outgrowth and growth cone turning (Aglah et al., 2008; Christensen et al., 2003; Kopperud et al., 2003; Murray et al., 2008; Murray et al., 2009). To determine the influence of Epac signaling on SGN neurites, we treated cultures with 8-pCPT-2'-O-Me-cAMP (8pCPT), a specific Epac agonist (Enserink et al., 2002; Kang et al., 2003; Murray et al., 2008). Mean neurite length was comparable in NT-3-positive control cultures and those treated with 200 μ M 8pCPT (Fig. 4) as well as lower or higher doses of the Epac agonist (1–500 μ M, not shown). Thus, the effect of cpt-cAMP signaling on SGN neurite length does not appear to be due to activation of Epac.

Given the biphasic response of SGN neurites to cpt-cAMP, we sought to determine if PKA signaling contributed to neurite length in the absence of PKA activators. Cultured SGNs were treated with Rp-cAMPS, a stereoisomer that functions as a specific PKA inhibitor and effectively blocks PKA at 1 mM in SGNs (Bok et al., 2003; Hansen et al., 2001). There was no significant difference in mean neurite length nor SGN survival in cultures treated with Rp-cAMPS compared with NT-3-positive control cultures (Fig. 4) suggesting that PKA signaling does not contribute to SGN neurite length in the absence of PKA activators.

Overexpression of GPKA, a constitutively active catalytic subunit of PKA tagged with green fluorescent protein (GFP), inhibited SGN neurite length

To further explore the effect of PKA signaling on SGN neurite length, we transiently transfected SGNs with GPKA, which consists of the catalytic domain of PKA fused with GFP, is constitutively active, and promotes SGN survival (Bok et al., 2003). Control cultures were transfected with GFP alone. Neurite length of transfected (GFP-positive) SGNs was determined as before (Atkinson et al., 2011; Hansen et al., 2007). SGNs transfected with GPKA had significantly shorter neurites compared to those transfected with GFP ($p < 0.001$, Mann-Whitney rank sum test) (Fig. 5). Although most SGNs expressing GPKA did not extend a long dominant neurite typical of SGNs transfected with GFP or untransfected SGNs, many extended multiple short processes (Fig. 5B,C), reminiscent of a

few of the SGNs treated with 10 mM cpt-cAMP (Fig 1D). As previously shown, expression of GPKA significantly increased SGN survival (Fig. 5, $p<0.05$, Student's t-test).

To determine whether the extent of PKA activity would inversely correlate with neurite length we took advantage of the fact that the intensity of GFP fluorescence provides an indication of the relative expression level of the constitutively active PKA subunit in the GPKA construct. We quantified the GFP fluorescence intensity in a subset of SGNs transfected with GFP or GPKA and plotted this intensity relative to neurite length (Fig. 5). There was no significant difference in the mean GFP intensity in GFP-transfected SGNs (179 ± 36 , arbitrary units, mean $\pm$ SD) compared with GPKA transfected SGNs (174 ± 29 , mean $\pm$ SD, $p=0.721$ Student's two-tailed t-test). In SGNs expressing GFP, there was no significant correlation between neurite length and GFP fluorescence intensity (Pearson's correlation coefficient, $r=-0.19$). In contrast, neurite length was inversely proportional to GFP fluorescence intensity in SGNs expressing GPKA ($r=-0.50$, $p<0.001$). Thus, those SGNs with higher levels of PKA expression, and by extension, activity tended to have the shortest neurites. These data further support the notion that high levels of PKA activity inhibit SGN neurite length.

Cytoplasmic or nuclear targeted GPKA inhibited SGN neurite length

PKA phosphorylates both cytoplasmic and nuclear targets (Shabb, 2001). In SGNs, restricting localization GPKA to the cytoplasm by addition of the nuclear export signal (nes) of mitogen-activated protein kinase kinase (MEK, EALQKKLEELELDE) is sufficient to promote survival, while targeting of GPKA to the nucleus with the addition of the nucleus localization signal (nls) of SV40 large T antigen (PPKKKRKVPK) fails to promote survival (Bok et al., 2003). To determine whether cytoplasmic or nuclear PKA substrates account for the inhibition of neurite length by PKA, SG cultures were transfected with GPKAnes or GPKAnls. Cultures were maintained in the presence of NT-3 to stimulate neurite growth. Both GPKAnes and GPKAnls significantly inhibited SGN neurite length compared to cultures transfected with GFP (Fig. 6). GPKAnes, but not GPKAnls, increased SGN survival, consistent with the observation that cytoplasmic PKA activity promotes SGN survival. Despite comparable inhibition of neurite length in SGNs transfected with GPKAnes and GPKAnls, SGNs transfected with these constructs displayed significant morphologic differences (Fig. 6). The vast majority of SGNs expressing GPKAnls ($85\pm8\%$, mean $\pm$ SEM) failed to extend any process (Fig. 6). Conversely, most SGNs expressing GPKAnes ($73\pm11\%$) extended many short, fine processes (>5 /neuron) that failed to develop into a dominant neurite similar to those expressing GPKA (Fig. 5).

Activation of phosphatidylinositol 3-kinase (PI3-K), but not c-Jun N-terminal kinase (JNK), partially rescued neurite length in SGNs expressing GPKA

Activation of PI3-K or JNK are independently sufficient to increase SGN neurite length (Atkinson et al., 2011; Renton et al., 2010). We asked if activation of either of these could rescue neurite length in SGNs with activated PKA. We co-transfected SGNs with GPKA and either P110, a constitutively active PI3-K subunit, MKK-JNK1, a constitutively active JNK isoform, or empty plasmid in a 1:1 ratio. In this case the cultures were maintained in the absence of neurotrophic factors. We have previously shown that P110 and MKK-JNK1 each increase SGN neurite length (Atkinson et al., 2011; Renton et al., 2010). Expression of P110, but not MKK-JNK1 significantly increased neurite length in SGNs expressing GPKA ($p<0.05$) (Fig. 7). However, the mean neurite length in SGNs expressing GPKA and P110 was still lower than the mean neurite length of SGNs expressing GFP and maintained in NT-3 (Fig. 5). Thus, PI3-K activity partially rescues neurite length in SGNs with activated PKA.

Inhibition of PKA failed to rescue SGN neurite length in depolarizing conditions

Membrane depolarization by elevated extracellular potassium promotes SGN survival, in part, by activating PKA (Bok et al., 2003; Hansen et al., 2001). Similar to higher levels of PKA activity, membrane depolarization also inhibits SGN neurite length (Roehm et al., 2008). To determine if activation of PKA contributes to the reduction of neurite length by membrane depolarization, we assayed neurite length in cultures treated with 30 or 80 mM extracellular K^+ (30K, 80K) in the presence or absence of Rp-cAMPS (1 mM). Treatment with Rp-cAMPS failed to prevent the reduction of SGN neurite length by 30K or 80K implying that activation of PKA does not account for the reduction of neurite length by depolarization. To further confirm this observation, we transfected SGNs with the GPKI plasmid that encodes a GFP-tagged PKA inhibitory peptide (Bok et al., 2003). GPKI has previously been shown to effectively block PKA signaling in SGNs (Bok et al., 2003). Control cultures were transfected with GFP. Similar to treatment with Rp-cAMPS, GPKI failed to rescue neurite length in depolarized SGNs, confirming that activation of PKA does not account for the inhibitory effects of membrane depolarization on SGN neurite length.

DISCUSSION

Following hair cell loss, the peripheral fibers of SGNs degenerate followed by a gradual loss of the neurons themselves. In humans, the peripheral fibers likewise degenerate although the neuronal loss occurs more slowly (Linthicum et al., 2009; Nadol, 1990; Zimmermann et al., 1995). This neural degeneration likely contributes to the poor spatial and temporal resolution provided by cochlear implants (Gillespie et al., 2005; Pettingill et al., 2007; Roehm et al., 2005; Xu et al., 2008); regrowth of the peripheral fibers to be in close proximity or even contact the stimulating electrodes will likely reduce activation thresholds and may allow for more independent channels (Rubinstein, 2004; Xu et al., 2008). Neural degeneration also complicates efforts to restore auditory perception by inducing hair cell regeneration. Hence, there is great interest in identifying factors and therapeutic strategies to prevent SGN degeneration and to promote fiber regeneration towards stimulating electrodes and possibly to new or regenerated hair cells if hair cell renewal efforts become feasible in the future (Boer et al., 2009; Gillespie et al., 2005; Pettingill et al., 2007; Roehm et al., 2005). Intracochlear application of neurotrophic factors enhances the regrowth of peripheral fibers in deafened cochleae (Miller et al., 2007; Shibata et al., 2010; Wise et al., 2010). Manipulation of other signals that influence SGN survival and neurite growth, in addition to those recruited by neurotrophic factors, may further enhance the regrowth of SGN peripheral fibers.

The second messenger cAMP exerts dramatic effects on neural differentiation and regeneration and several strategies to promote fiber regeneration include efforts to modulate cAMP levels. Here we have begun to explore the influence of cAMP signaling on SGN neurite length. Taken together our results demonstrate that cAMP exerts a biphasic effect on SGN neurite length, increasing length at lower concentrations while reducing length at higher concentrations. FSK also increases SGN neurite length; however, in contrast to cpt-cAMP, we did not note a concentration above which FSK reduce neurite length. FSK increases intracellular cAMP by activating adenylate cyclase (Seamon et al., 1983). Thus, the rise in cAMP in cells treated with FSK is limited to those subcellular regions where adenylate cyclase is localized (e.g. cell membrane), by the maximal activity level of adenylate cyclase, and by cAMP degradation by cyclic nucleotide phosphodiesterases. Conversely, treatment with cpt-cAMP increases cAMP throughout the cell. Such differences may account for the reduction of neurite length with increasing cpt-cAMP doses but not FSK.

Prior work in embryonic chick dorsal root ganglion neurons suggests that growth cones require a balance between cAMP levels and integrin function to maintain motility and prevent collapse (Lemons et al., 2006). Neurons plated on low or high concentrations of laminin extend growth cones at similar speeds, yet have distinct levels of integrin expression, integrin activation and intracellular cAMP levels. Increasing cAMP enhanced growth cone motility on low concentrations of laminin yet induced growth cone collapse and retraction on high concentrations of laminin but not fibronectin (Lemons et al., 2006). The neurons cultured on high laminin concentrations demonstrated increased endogenous cAMP levels. These observations are consistent with our finding of a biphasic response of SGN neurites to cAMP, with higher levels reducing neurite length. Nevertheless, this biphasic response in SGNs was not substrata-dependent occurring on laminin, fibronectin, or tenascin C implying that the response occurs independent of the pattern of integrin receptor activity.

We also found that elevation of cAMP decreased SGN neurite branching. Recent work in embryonic rat hippocampal neurons demonstrated that elevation of cAMP in a neurite inhibits the growth of the other neurites by modulating cAMP and cGMP levels within the distant branches (Shelly et al., 2010). Such competitive inhibition of neurite process outgrowth arising from long-distance cAMP signaling represents one possible mechanism whereby elevated cAMP could reduce SGN neurite branching. In addition to reducing neurite length, we found that a high level of PKA activity prevented formation of a dominant neurite although neurons expressing GPKA or GPKAnes extended many fine neurites suggesting that PKA activity suppresses formation of dominant neurites and perhaps neuritogenesis.

Intracellular cAMP levels also determine the response of growth cones of in a variety of neurons, including rat dorsal root and retinal ganglion neurons and *Xenopus* spinal neurons, to guidance cues (Cai et al., 2001; Ming et al., 1997; Song et al., 1998; Song et al., 1997). In rat dorsal root ganglion neurons, increased levels of cAMP favor attraction while low cAMP levels results in repulsion (Cai et al., 2001; Murray et al., 2008; Murray et al., 2009). Decreased endogenous intracellular cAMP levels during development likely underlies the developmental switch in growth cone responsiveness to guidance cues (Cai et al., 2001). Embryonic neurons have high levels of intracellular cAMP and central myelin components such as myelin associated glycoprotein (MAG) promote neurite growth while in postnatal neurons endogenous cAMP levels are low and MAG switches from being growth promoting in embryos to repulsive and growth inhibitory postnatally (Murray et al., 2009). Increasing cAMP levels in postnatal neurons helps overcome the inhibitory effect of repulsive cues of central glia and myelin. Recent work demonstrated that central glia inhibit neurite growth from postnatal SGNs and that elevation of cAMP overcomes this inhibitory effect (Jeon et al., 2011). In this study, cultures were derived from postnatal neurons and, in this case, whether cAMP promotes or inhibits growth depends on its concentration.

cAMP activates PKA and Epac; both of which have been shown to regulate neurite growth in PC12 cells and a variety of neurons including rat motor and sensory neurons and *Xenopus* spinal neurons (Aglah et al., 2008; Christensen et al., 2003; Han et al., 2007; Kopperud et al., 2003; Murray et al., 2008; Murray et al., 2009). In chick DRGs, the embryonic attraction in response to cAMP is mediated by Epac, while repulsion is mediated by PKA (Murray et al., 2009) whereas in *Xenopus* neurons PKA activity promotes attraction (Han et al., 2007). To address the possibility that Epac may influence SGN neurite growth, we treated cultures with a cAMP analog that specifically activates Epac, 8-pCPT. The selective Epac activator did not increase or reduce postnatal SGN neurite length suggesting that both the progrowth and the inhibitory effects of cAMP are likely to be mediated by PKA.

High levels of PKA activity inhibit SGN neurite length

Our data suggest that high levels of PKA activity inhibits SGN neurite growth since transfection of constitutively active PKA isoforms drastically reduces neurite length. PKA phosphorylates cytoplasmic and nuclear targets. In SGNs cytoplasmic but not nuclear-targeted PKA promotes survival demonstrating that PKA phosphorylates cytoplasmic targets to promote SGN survival (Bok et al., 2003). In *Xenopus* embryonic neurons PKA promotes neurite growth; an effect that is attributed to phosphorylation of synapsin, a cytoplasmic protein (Kao et al., 2002). Here we used active PKA isoforms excluded from the nucleus (cytoplasmic) or targeted to the nucleus to determine the subcellular location of PKA on SGN neurite growth. Both cytoplasmic and nuclear-targeted PKA reduced SGN neurite length. However, there were morphological differences in neurons expressing GPKAnls and GPKAnes. SGNs expressing GPKAnes extended multiple fine neurites that failed to develop into a long dominant neurite. Some of the SGNs treated with 10 mM cpt-cAMP exhibited a similar morphology. However, most SGNs expressing GPKAnls failed to develop neurites. These results raise the possibility that nuclear-targeted PKA abrogates neuritogenesis whereas cytoplasmic-targeted PKA likely prevents formation of a dominant neurite and inhibits neurite extension.

Activity dependent signals promote SGN survival and inhibit neurite length

Chronic membrane depolarization *in vitro* activates several Ca^{2+} -dependent signaling cascades including PKA, Ca^{2+} /calmodulin-dependent protein kinase (CaMK) II, and CaMKIV to support SGN survival (Bok et al., 2003; Bok et al., 2007; Hansen et al., 2001; Hansen et al., 2003; Hegarty et al., 1997). Chronic membrane depolarization also inhibits SGN neurite length (Roehm et al., 2008). Further, CaMKII and PKA, but not CaMKIV, inhibit neurite growth (Hansen et al., 2003). Previously we found that CaMKII activity does not contribute to the reduction of neurite length by chronic membrane depolarization (Roehm et al., 2008). Here we tested the possibility that activation of PKA accounts for the reduction of SGN neurite length by chronic membrane depolarization. Inhibition of PKA with Rp-cAMPS or overexpression of PKI failed to rescue neurite length in depolarized neurons. Thus, similar to CaMKII, PKA is activated by depolarization to promote SGN survival and reduces SGN neurite length. However, neither CaMKII nor PKA account for the reduction of neurite length by chronic membrane depolarization. In addition to Ca^{2+} -dependent kinases, depolarization also activates the Ca^{2+} -dependent protease, calpain, and inhibition of calpain partially rescues neurite length in depolarized SGNs (Roehm et al., 2008).

Interaction of PKA with PI-3 Kinase and JNK

Neurotrophins such as BDNF and NT-3 promote SGN survival and neurite growth by activating Trk receptors and downstream signaling cascades including PI-3K/Akt and mitogen activated protein kinases such as Erk and JNK (Aletsee et al., 2001; Atkinson et al., 2011; Hansen et al., 2001; Renton et al., 2010). Activation of PI-3K and JNK each independently increase SGN neurite length (Atkinson et al., 2011; Renton et al., 2010). There are 3 isoforms of JNK, 1, 2, and 3 and each partially contributes to SGN neurite length (Atkinson et al., 2011). To begin to understand the influence of PKA on intracellular signals that promote SGN neurite growth, we asked whether constitutively active isoforms of PI3-K or JNK could rescue neurite length in SGNs expressing GPKA. We found that constitutively active JNK1 failed to rescue SGN neurite length. By contrast, constitutively active PI3-K partially rescued neurite length in SGNs expressing PKA, suggesting that PI3-K functions downstream and/or independent of PKA to promote SGN neurite length.

Summary

Modulation of cAMP levels and activation of PKA, either directly by infusion of molecules into the cochlea and/or exogenous gene transfer or indirectly by activating Ca²⁺-dependent signals, represents a potential therapeutic approach to support SGN survival. To the extent that the response of cultured SGN neurites recapitulates the response of SGN peripheral and/or central fibers *in vitro*, raising intracellular cAMP to appropriate levels may also enhance regrowth of SGN peripheral fibers and even enhance regeneration of central SGN fibers across central glial boundaries (Jeon et al., 2011). However, our results suggest that such therapies require careful evaluation and titration. Elevated cAMP/PKA activity, like many other signals that promote survival including chronic membrane depolarization (Roehm et al., 2008), Bcl-2 (Hansen et al., 2007), and CaMKII (Hansen et al., 2003), reduces neurite length potentially complicating approaches aimed at preventing SGN apoptosis while simultaneously promoting regeneration of auditory nerve fibers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
CaMK	Ca ²⁺ /calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
DMSO	dimethyl sulfoxide
EPAC	Exchange Protein directly Activated by Cyclic AMP
FSK	forskolin
GFP	green fluorescent protein
JNK	c-Jun N-terminal kinase
MAG	myelin associated glycoprotein
MEK and MKK	mitogen-activated protein kinase kinase
nes	nuclear export signal
NF200	neurofilament 200
nls	nucleus localization signal
NT-3	neurotrophin-3
PI3-K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKI	protein kinase A inhibitory peptide
SEM	standard error of the mean
SGN	spiral ganglion neuron

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Highlights

1. cAMP exerts a biphasic effect on SGN neurite length.
2. The effect of cAMP on SGN neurite length is not substrate dependent.
3. PKA activity in the cytoplasm and in the nucleus inhibits SGN neurite length.
4. PI3-K signaling partially rescues neurite length in SGNs with elevated PKA activity.
5. PKA does not contribute to the inhibition of neurite length by depolarization.

Figure 1.

cAMP exerts a biphasic effect on SGN neurite length. **A.** Representative images of spiral ganglion cultures maintained in NT-3 or NT-3 with cpt-cAMP (1 mM, 10mM) as indicated for 48 h and labeled with anti-NF200. Scale bars=100 μ m and 25 μ m (bottom right panel). **B.** Mean neurite length in SG cultures maintained in NT-3-negative control medium, NT-3 (50 ng/ml, NT-3-positive control), NT-3 with cpt-cAMP (0.1–10 mM), or cpt-cAMP (1 mM). Each condition was performed in duplicate (2 wells/repetition) and at least 60 neurites were measured for each condition. The conditions were repeated in at least three separate culture preparations (≥ 6 wells total). The total number of neurites scored in each condition is indicated in each bar. * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison. **C.** Normalized average SGN survival in cultures maintained in NT-3-negative control medium, NT-3 (50 ng/ml, NT-3-positive control), NT-3 with cpt-cAMP (0.1–10 mM), or cpt-cAMP (1 mM). 100% represents survival in NT-3. * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison compared to NT-3-positive control. **D.** Mean neurite length in SG cultures maintained NT-3 with or without forskolin (FSK, 0.1–10 μ M). * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison compared to NT-3-positive control. **E.** Normalized average SGN survival in SG cultures maintained NT-3 with or without forskolin (FSK, 0.1–10 μ M). * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison compared to NT-3-positive control.

Figure 2.

Influence of cAMP on neurite branching. **A.** Mean primary branching index in cultures maintained in control medium, NT-3 with or without cpt-cAMP (0.1–10 mM), or cpt-cAMP (1 mM). **B.** Mean secondary branching index in cultures maintained in control medium, NT-3 with or without cpt-cAMP (0.1–10 mM), or cpt-cAMP (1 mM). * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison compared with NT3-positive control cultures. * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison compared with NT3-negative control cultures. ** $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison compared with NT3-negative control cultures.

Figure 3.

The biphasic effect of cpt-cAMP on SGN neurite length is not dependent on culture substrate. Mean neurite length in SG cultures plated on fibronectin or tenascin C and maintained in NT-3 with or without cpt-cAMP (1, 10 μ M) or forskolin (FSK, 1 μ M). The total number of neurites scored in each condition is indicated in each bar. * $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison.

Figure 4.

8-pCPT-2'-O-Me-cAMP, an Epac selective cAMP analog, does not alter SGN neurite length or survival. **A.** Mean neurite length of SGNs treated with 8-pCPT-2'-O-Me-cAMP (200 μM) or Rp-cAMPS (1 mM) in the presence of NT-3 was not significantly different from control SGNs maintained in NT-3 ($p > 0.05$, one way ANOVA). The total number of neurites scored in each condition is indicated in each bar. **B.** Normalized average SGN survival in cultures treated with 8-pCPT-2'-O-Me-cAMP (200 μM) or Rp-cAMPS (1 mM) in the presence of NT-3 was not significantly different from control SGNs maintained in NT-3 ($p > 0.05$, one way ANOVA).

Figure 5.

Expression of GPKA, a constitutively active PKA isoform, reduces SGN neurite length.

A&B. SG cultures transfected with GFP (A, green) or GPKA (B, green) and immunostained with anti-NF200 (red). Scale bar=100 μ m. **C.** Higher magnification image of a SGN transfected with GPKA and immunostained with anti-NF200. Scale bar=25 μ m. There are a few transfected non-neuronal cells (GFP-positive, NF200-negative) in each panel of A, B, and C. **D.** Mean neurite length of SGNs expressing GFP or GPKA in the presence of NT-3 (* p <0.001, Mann-Whitney rank sum test). The total number of neurites scored in each condition is indicated in each bar. **E.** Normalized average survival of SGNs expressing GFP or GPKA in the presence of NT-3 (* p <0.05, Student's t-test). **F&G.** Correlation of neurite length with expression level based on GFP fluorescence intensity in arbitrary units in a subset of SGNs expressing GFP (F) or GPKA (G). Pearson's correlation coefficients were r =-0.19 for GFP and r =-0.50 (p <0.001) for GPKA.

Figure 6.

GPKA reduces SGN neurite length regardless of nuclear targeting. **A.** Mean neurite length of SGNs expressing GFP, GPKA_{nes}, or GPKA_{nl}s in the presence of NT-3 (* $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison). The total number of neurites scored in each condition is indicated in each bar. **B.** Representative images of SGNs transfected with GPKA_{nl}s and immunostained with anti-NF200 (red). Scale bar=25 μm. There is also a transfected non-neuronal cell (GFP-positive, NF200-negative). **C.** Normalized average survival of SGNs expressing GFP, GPKA_{nes}, or GPKA_{nl}s in the presence of NT-3 (* $p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison).

Figure 7.

Expression of constitutively active PI3-K, but not constitutively active JNK, isoforms rescues neurite length in SGNs expressing GPKA. **A.** SGN co-transfected with P110 and GPKA and immunostained with anti-NF200 (red). Scale bar=25 μ m. A portion of the neurite travels in and out of the focal plane. There are a few transfected non-neuronal cells (GFP-positive, NF200-negative). **B.** Mean neurite length of SGNs co-transfected with GPKA and either empty vector, P110, or MKK-JNK1 (* p <0.05 by one way ANOVA followed by a Holm-Sidak post-hoc comparison). The total number of neurites scored in each condition is indicated in each bar. **C.** Normalized average survival of SGNs transfected with GPKA, GPKA+P110, or GPKA+MKK-JNK1 (* p <0.05, one way ANOVA followed by a Holm-Sidak post-hoc comparison).

Figure 8.

PKA activity is not required for the reduction of neurite length by depolarization. **A.** Spiral ganglion cultures were maintained in NT-3, NT-3+30K, or NT-3+80K with or without Rp-cAMPS (1 μ M) for 48 h as indicated. Mean neurite length in cultures maintained NT-3+30K+Rp-cAMPS and NT-3+80K+Rp-cAMPS was not significantly different ($p > 0.05$, one way ANOVA) from cultures maintained in NT-3+30K and NT-3+80K, respectively. **B.** Spiral ganglion cultures were transfected with expression plasmids encoding GFP-tagged PKA inhibitory peptide (GPKI) or GFP. Cultures were maintained in NT-3, NT-3+30K, or NT-3+80K for 48 h after transgene expression as indicated. Mean neurite length in cultures maintained in NT-3+30K+GPKI and NT-3+80K+GPKI were not significantly different ($p > 0.05$) from mean neurite length in cultures maintained in NT-3+30K+GFP and NT-3+80K+GFP, respectively, but both were significantly different than NT-3+GFP ($p < 0.05$, one way ANOVA followed by a Holm-Sidak post-hoc comparison). The total number of neurites scored in each condition is indicated in each bar.