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Research paper

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 *E*xchange *P*rotein directly *A*ctivated by *C*ylic 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 promote rather than inhibit nerve fiber regeneration.

1. 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 and Shepherd, 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, 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,

Abbreviations: ANOVA, analysis of variance; BDNF, brain derived neurotrophic factor; CaMK, Ca2+/calmodulin-dependent protein kinase; cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; EPAC, *E*xchange *P*rotein directly *A*ctivated by *C*ylic 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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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 and Ohlemiller, 2010; Dodson and Mohuiddin, 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 and Fayad, 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 and Pfingst, 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 and Raz, 2004; Gillespie and Shepherd, 2005; Pettingill et al., 2007; Richardson et al., 2009; Roehm and Hansen, 2005; Staecker and Garnham, 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 and Roisen, 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, *E*xchange *P*rotein directly *A*ctivated by *C*ylic AMP (Epac) (Aglah et al., 2008; Christensen et al., 2003; Han et al., 2007; Kopperud et al., 2003; Murray and Shewan, 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.

2. Methods

2.1. 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 N_2 (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% CO_2. 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).

2.2. 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, 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, 2003; Zha et al., 2001). Approximately 5–10% of non-neuronal cells are also transfected.

2.3. 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,