Published in final edited form as:

Neuroscience. 2013 August 15; 245: 74–89. doi:10.1016/j.neuroscience.2013.04.016.

Cyclic AMP Stimulates Neurite Outgrowth of Lamprey Reticulospinal Neurons without Substantially Altering Their Biophysical Properties

Timothée Pale¹, Emily B. Frisch¹, and Andrew D. McClellan^{1,2}

¹Division of Biological Sciences, University of Missouri, Columbia, MO 65211-6190

²Interdisciplinary Neuroscience Program, University of Missouri, Columbia, MO 65211-6190

Abstract

Reticulospinal (RS) neurons are critical for initiation of locomotor behavior, and following spinal cord injury (SCI) in the lamprey, the axons of these neurons regenerate and restore locomotor behavior within a few weeks. For lamprey RS neurons in culture, experimental induction of calcium influx, either in the growth cone or cell body, is inhibitory for neurite outgrowth. Following SCI, these neurons partially downregulate calcium channel expression, which would be expected to reduce calcium influx and possibly provide supportive conditions for axonal regeneration. In the present study, it was tested whether activation of second messenger signaling pathways stimulates neurite outgrowth of lamprey RS neurons without altering their electrical properties (e.g. spike broadening) so as to possibly increase calcium influx and compromise axonal growth. First, activation of cAMP pathways with forskolin or dbcAMP stimulated neurite outgrowth of RS neurons in culture in a PKA-dependent manner, while activation of cGMP signaling pathways with dbcGMP inhibited outgrowth. Second, neurophysiological recordings from uninjured RS neurons in isolated lamprey brain-spinal cord preparations indicated that dbcAMP or dbcGMP did not significantly affect any of the measured electrical properties. In contrast, for uninjured RS neurons, forskolin increased action potential duration, which might have increased calcium influx, but did not significantly affect most other electrical properties. Importantly, for injured RS neurons during the period of axonal regeneration, forskolin did not significantly alter their electrical properties. Taken together, these results suggest that activation of cAMP signaling by dbcAMP stimulates neurite outgrowth, but does not alter the electrical properties of lamprey RS neurons in such a way that would be expected to induce calcium influx. In conclusion, our results suggest that activation of cAMP pathways alone, without compensation for possible deleterious effects on electrical properties, is an effective approach for stimulating axonal regeneration of RS neuron following SCI.

Keywords

axonal regeneration; spinal cord injury; cAMP; PKA; cGMP

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Address correspondence to: Andrew D. McClellan, 114 Lefevre Hall, Division of Biological Sciences, University of Missouri, Columbia, MO 65211-6190, Tel: (+1) 573-882-1447, Fax: (+1) 573-884-5020, McclellanA@missouri.edu.

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1. INTRODUCTION

In all vertebrates, reticulospinal (RS) neurons in the brain activate spinal central pattern generators (CPGs) to initiate locomotor behavior. For example, continuous stimulation of RS neurons is sufficient for initiation of locomotion in a wide variety of vertebrates: lamprey (Jackson et al., 2007); stingray (Livingston and Leonard, 1990); dogfish (Grillner and Wallén, 1984); fish (Uematsu and Todo, 1997); birds (Steeves et al., 1987); and mammals (Ross and Sinnamon, 1984; Garcia-Rill and Skinner, 1987). In addition, in some vertebrates, RS neurons also have been shown to be necessary for initiation of locomotion (Shefchyk et al., 1984; Bernau et al., 1991; Marlinski and Voitenko, 1992; Paggett et al., 2004).

Following severe spinal cord injury (SCI) in higher vertebrates (birds and mammals), there is very little axonal regeneration of injured RS neurons and permanent loss of voluntary motor control below the lesion site (Bradbury and McMahon, 2006). In contrast, following SCI in “lower” vertebrates, such as the lamprey, fish, and certain amphibians, locomotor behaviors recover after a few weeks (reviewed in McClellan, 1998, 2012). In the lamprey, as in other lower vertebrates, the CNS is a permissive environment such that the axons of injured RS neurons regenerate back into the spinal cord (Davis and McClellan, 1994a) and make functional connections with spinal neurons (Mackler and Selzer, 1987) to restore locomotor function (Davis et al., 1993).

At 2–3 weeks following SCI in the lamprey and during axonal regeneration, injured RS neurons display dramatic changes in their properties compared to uninjured neurons (McClellan et al., 2008). For example, in response to applied current pulses, uninjured neurons fire a smooth train of spikes, while injured RS neurons fire a single, short burst or short repetitive bursts of action potentials as a result of reduced electrical excitability (McClellan, 2003, 2009). In addition, for uninjured RS neurons the main depolarizing phase of action potentials is followed by afterpotentials with three components (McClellan et al., 2008): (a) fast afterhyperpolarization (fAHP) that is due to fast potassium channels; (b) after depolarization (ADP), which is not abolished by calcium channel blockers and may be due to persistent sodium channels; and (c) slow AHP (sAHP) that is mediated by high-voltage activated (HVA) calcium channels and calcium-activated potassium channels (K_{Ca}). For injured RS neurons at early recovery times, the ADP and sAHP are absent. Finally, injured RS neurons exhibit a reduction in mRNA levels for both HVA calcium channels and K_{Ca} channels (McClellan et al., 2008). Reduced excitability and a decrease in calcium channels in injured RS neurons would be expected to reduce or limit calcium influx. Furthermore, for lamprey RS neurons in culture, manipulations that induce calcium influx, either in growth cones or cell bodies, inhibit neurite outgrowth (Ryan et al., 2007). Interestingly, calcium influx in a single growth cone only inhibits outgrowth of the parent neurite, while influx in the cell body inhibits all of the associated neurites. In general, calcium influx can compromise or inhibit neurite outgrowth of neurons in culture (Kater and Mills, 1991; Bandtlow et al., 1993; Moorman and Hume, 1993) as well as axonal regeneration following neuronal injury (Enes et al., 2010). For lamprey RS neurons, it is hypothesized that following SCI, changes in the electrical properties of these neurons reduce calcium influx and maintain intracellular calcium levels in a range that is permissive for axonal regeneration (Ryan et al., 2007; McClellan et al., 2008).

Following SCI, second messengers can promote axonal regeneration, but the mechanisms are poorly understood. For example, activation of cAMP signaling can support axonal regeneration of Mauthner neurons and recovery of startle behavior in spinal cord-lesioned zebrafish (Bhatt et al., 2004). For spinal cord-injured mice, stimulation of cAMP pathways in the spinal cord, brainstem, or sensorimotor cortex overcomes myelin-associated inhibitors

and promotes axonal outgrowth (Pearse et al., 2004). In rats following spinal cord injury, rolipram, which inhibits breakdown of endogenous cAMP, enhances axonal regeneration and promotes functional recovery (Nikulina et al., 2004). Activation of cGMP signaling enhances regeneration of retinal axons in the injured goldfish optic nerve (Koriyama et al., 2009) and axons in the injured locust ventral nerve cord (Stern and Bicker, 2010).

Second messenger signaling can regulate neurite outgrowth of neurons in culture from a variety of animals. For example, for *Xenopus* spinal neurons, activation of cAMP or cGMP signaling can overcome specific repulsive factors and stimulate neurite outgrowth (Song et al., 1998; Togashi et al., 2008). For postnatal rat retinal or sensory neurons in culture, activation of cAMP signaling negates the inhibitory effects of myelin-associated glycoprotein and promotes neurite outgrowth (Cai et al., 2001). Moreover, rolipram stimulates neurite outgrowth of cultured rat DRG neurons grown on a myelin substrate (Nikulina et al., 2004). In contrast, forskolin and dbcAMP both suppress neurite elongation for *Helisoma* neurons (Mattson et al., 1988).

In addition to modulating axonal outgrowth, second messengers also can regulate the electrophysiological properties of neurons. For example, cAMP signaling decreases potassium currents in several types of neurons (Herness et al., 1997), and in many cases this leads to action potential broadening (Parker et al., 1997; Hochner and Kandel, 1997; Goldsmith and Abrams, 1992; Ansanay et al., 1995). However, in *Helix* neurons application of forskolin lengthens action potentials through inactivation of voltage-gated K⁺ channels, but in a cAMP-independent manner (Watanabe and Gola, 1987). In addition, activation of cGMP pathways can have variable effects on electrical properties. For example, activation of cGMP signaling reduces voltage-gated calcium currents in a PKG-dependent fashion in human neuroblastoma cells (D'Ascenzo et al., 2002) but decreases calcium currents in a PKG-independent manner for guinea pig hippocampus neurons (Doerner and Alger, 1988).

Although second messengers, specifically cAMP, have been used to treat SCI, and the cellular mechanisms for their growth-promoting actions have been investigated, studies have not directly focused on the effects of these signaling molecules on RS neurons. Yet, these are the key neurons whose axonal regeneration following SCI is important for recovery of locomotor behavior. The present study tested, for the first time, if activation of second messenger pathways stimulates neurite outgrowth of lamprey RS neurons in culture. In addition, the effects of second messenger signaling on the electrical properties of lamprey RS neurons were tested in isolated brain-spinal cord preparations. In particular, the experiments tested whether these agents alter the electrical properties of RS neurons in such a way that might increase calcium influx and partially compromise an agent's growth promoting actions. For example, if activation of second messenger signaling were to increase action potential duration of injured RS neurons, this very likely would increase calcium influx (McCobb and Beam, 1991; Toth and Miller, 1995), which is known to compromise neurite outgrowth in culture (Kater, 1991; Bandtlow et al., 1993; Moorman and Hume 1993; Ryan et al., 2007) as well as axonal regeneration following neuronal injury (Enes et al., 2010). Preliminary results from this study have been presented in abstract form (Pale et al., 2011).

2. EXPERIMENTAL PROCEDURES

2.1 Animal Care

Larval sea lampreys (*Petromyzon marinus*) were used for both cell culture and neurophysiological experiments and were maintained in ~10 liter aquaria at 23°C. The procedures in this study have been approved by the Animal Use and Care Committee at the University of Missouri.

2.2 Experimental Rationale

The main purpose of the study was to examine the effects of second messengers (mainly cAMP) on neurite outgrowth and biophysical properties of lamprey RS neurons. First, the effects of agents on neurite outgrowth of lamprey RS neurons were best performed in cell culture because this allowed manipulations not easily performed *in vivo*. However, it is common knowledge that the specific state of neurons in culture often is not entirely known, and some of the properties of these neurons might not be directly comparable to the properties of neurons within an intact nervous system. Thus, in the current study some of the properties of lamprey RS neurons in culture might not be directly comparable to those recorded from these neurons within isolated brain-spinal cord preparations (see below). Second, the effects of agents on biophysical properties of RS neurons were best determined using isolated brain-spinal cord preparations, which allowed a comparison of drug effects on both injured lamprey RS neurons that were just starting to regenerate their axons through the injury site (Davis and McClellan, 1994b) and uninjured neurons (see 2.4.1 Spinal cord lesions, below). In cell culture, it was not possible to compare the effects of cAMP activation on both uninjured and injured neurons, because the procedure of dissociating the neurons probably resulted in a certain degree of injury.

2.3 Cell Culture

With our experimental procedures, lamprey RS neurons in culture have diameters that ranged between ~11–55 μm , and thus probably are small, unidentified neurons and not the large, identified Müller and Mauthner cells (Ryan et al., 2007). In culture, these unidentified RS neurons have variable growth cone shapes, ranging from simple bulb-like structures to somewhat more complex shapes, including some lamellipodia and filopodia (Ryan et al., 2007 and in preparation).

The effects of second messenger signaling on neurite outgrowth were tested on lamprey RS neurons in culture, which allowed manipulations not easily performed *in vivo*. Animals ($n = 37$) were anesthetized in tricaine methanesulphonate (~200 mg/l; MS222; Crescent Research Chemicals; Phoenix, AZ), and the spinal cords were exposed and transected at 20% body length (BL, normalized distance from the anterior tip of the head). A small piece of Gelfoam (Upjohn; Kalamazoo, MI) soaked in a solution of 8% 1,10-dioctadecyl-3,3',30,30'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes; Eugene, OR) in dimethylformamide (DMF; Sigma Chemical; St. Louis, MO) was applied between the severed ends of the spinal cord to retrogradely label descending brain neurons, as previously described (Ryan et al., 2007). The incision was closed and sealed with cyanoacrylate (Super Glue Gel; Loctite Company, Rocky Hill, CT), and animals were returned to aquaria to recover for ~2–4 weeks.

Following retrograde labeling, animals were re-anesthetized, brains were removed in sterile Ringer's solution and sometimes viewed with a fluorescence microscope to confirm the presence of DiI-labeled brain neurons. Brains were transected at the rostral diencephalon and caudal rhombencephalon, and then cut into blocks of ~200–500 μm . Descending brain neurons, ~80% of which are RS neurons (Davis and McClellan, 1994a), were dissociated and plated in culture dishes, as previously described (Ryan et al., 2007). Briefly, tissue blocks were digested in sterile collagenase solution (1.25 mg/ml; Sigma) for 30 min and subsequently in sterile protease solution (0.5 mg/ml; Sigma) for 16 min. The tissue was triturated, and isolated brain neurons were plated in cell culture Petri dishes with a poly-D-lysine substrate (Biocoat; Becton-Dickinson Labware, Bedford, MA) and containing 2 ml of custom, salt-balanced L15 media (Gibco, Life Technologies; Grand Island, NY) and 10% fetal bovine serum (Gibco). Media was maintained at ~23°C and changed every ~3 days.

Approximately 4–10 days post plating, several DiI-labeled RS neurons per culture dish with new neurites were selected for experiments. *In vivo*, RS neurons usually have several dendrites and a single axon (Rovainen, 1978, 1979), while in culture, the neurons have one or perhaps two neurites (Ryan et al., 2007). Thus, it is likely, although not proven, that the neurites are axons. If a neuron in culture had two processes, the longer process was selected for analysis. Some of the largest diameter neurons in culture (Ryan et al., 2007) might correspond to the large, identified RS neurons (Zhang et al., 2002). However, neuronal cell bodies in culture are attached to a flat surface, and thus, these cell body diameters might not be directly comparable to those of neurons within an intact brain.

Phase-contrast images of neuronal cell bodies and associated neurites were captured using time-lapse video microscopy (2–10 min interval) (Ryan et al., 2007) during a pre-control period of 120 min. Subsequently, during the experimental period (120 min), one of the following agents that affects second messenger signaling was added to the culture dish: 10 mM dbcAMP (Sigma); 100 μ M forskolin (Tocris Cookson, Ellisville, MO); 100 μ M IBMX (Sigma); 10 μ M H89 (Sigma); 10 μ M 1NM-PP1 (Calbiochem, La Jolla, CA); 30 μ M PKI(14–22) (PKA inhibitor fragment 14–22 amide, myristoylated; Calbiochem); or 10 mM dbcGMP (Sigma). Forskolin and 1NM-PP1 were made up as a 250X solution in DMSO, and 8 μ l of one of these solutions was added to the 2 ml culture dishes. The agents PKI(14–22) and H89 were made up at 100X concentration in water, and 20 μ l of one of these solutions was added to the culture dishes. The remaining agents were made up as a 2X solution in media, and 1 ml of one of these solutions was used to replace 1 ml of media in culture dishes. Control experiments indicate that 0.4% DMSO or solution changes using Ringer's solution or media did not significantly alter neurite outgrowth (data not shown; see Ryan et al., 2007).

Time-lapse images were displayed on a computer screen using custom image-digitizing software, x,y points were marked along neurites, and the rates of neurite outgrowth (μ m/hr) during pre-control (media; 120 min) and experimental (agent present; 120 min) periods were calculated, as previously described (Ryan et al., 2007). For lamprey RS neurons in culture, during the pre-control periods, some neurites initially were extending and others were retracting as their growth cones explored the environment. Therefore, in the present study, neurites were sorted into two groups, those that were extending or retracting during pre-control periods, and analyzed separately. The neurite outgrowth rates during pre-control and experimental periods were compared statistically with a paired t-test.

2.4 Neurophysiology

The effects of second messenger signaling on electrical properties were tested on lamprey RS neurons in brain-spinal cord preparations, which allow comparisons of drug effects on uninjured and injured neurons (see below). In the lamprey, RS neurons are located in the mesencephalic reticular nucleus (MRN) and also in the anterior (ARRN), middle (MRRN) and posterior (PRRN) rhombencephalic reticular nuclei (Fig. 1) (Rovainen, 1978, 1979). There are ~28 large uniquely identifiable RS neurons, called Müller and Mauthner cells, which have long descending axons that project to the caudal spinal cord (Rovainen, 1978; Davis and McClellan, 1994b). Müller cells are located in the MRN (M1–M3), ARRN (I1–I4), and MRRN (B1–B5), while the Mauthner cells (Mau, AM) are located in the MRRN (Fig. 1) (Rovainen, 1978).

In the present study, Müller and Mauthner cells were used as general representatives of lamprey RS neurons for the following reasons. First, although these large RS neurons do not appear to be necessary and sufficient for initiation of locomotion (Shaw et al., 2010), as is the case for smaller, unidentified RS neurons (Paggett et al., 2004; Jackson et al., 2007), Müller and Mauthner cells fire rhythmically during locomotor activity (Kasicki and Grillner,

1986; Zelinin, 2005). Also, some of these neurons appear to have direct or indirect inputs to spinal CPGs (Buchanan and Cohen, 1982). Second, in the present study, RS neurons were impaled before drug application (control) and often later re-impaled after drug application to the bath (experimental) to maximize the numbers of neurons that could be characterized in each experiment, and the large sizes of Müller and Mauthner cells made this approach practical.

2.4.1 Spinal cord lesions—Larval sea lampreys (~80–130 mm; n = 17 animals) were anesthetized, and the spinal cord was exposed at ~10% BL, which is ~2 mm caudal to the brain. Iridectomy scissors and fine forceps were used to make double spinal hemi-transections (HTs; Fig. 10A), to mimic the general lesion procedures for single, unilateral HTs that were used in previous studies (McClellan et al., 2008). The double HTs severed the axons of all RS neurons, including Müller and Mauthner cells. Animals recovered for 2–3 weeks, at which time some of the large RS neurons have just started to regenerate their axons through the spinal lesion site (Davis and McClellan, 1994b), and axotomy-induced changes in electrophysiological properties are most pronounced (McClellan et al., 2008). For example, at 3–6 weeks following spinal transections at 10% BL, a few of the large RS neurons can sometimes regenerate their axons for at least ~10 mm caudal to the lesion site, but it takes much longer for many of these neurons to regenerate their axons for this distance (Davis and McClellan, 1994b).

2.4.2 Isolated brain-spinal cord preparations and intracellular recordings—The brains and rostral spinal cords of normal lampreys (n = 25 animals) and animals with double HTs (n = 17) were removed, as described previously (Rouse et al., 1998; McClellan et al., 2008), and pinned dorsal side up in a small neurophysiological recording chamber containing oxygenated lamprey Ringer's solution (6–9°C; pH 7.4) (McClellan, 1990). For all experiments, a suction electrode was placed around the caudal end of the spinal cord (~20% BL) to monitor possible orthodromic responses elicited by stimulation of RS neuron cell bodies (Fig. 6A,8A,10A). For animals with double HTs, a suction electrode also was placed on the dorsal surface of the spinal cord approximately halfway between the brain and spinal lesions to monitor orthodromic responses above the injury sites (Fig. 10A) (McClellan et al., 2008).

Micropipettes filled with 5 mM potassium acetate (~50–100 M Ω) were used to record from uninjured and axotomized RS neurons with either conventional current clamp ("bridge mode") or discontinuous current clamp (DCC) (fs ~ 6 kHz). All data were stored on tape (Neurodata DR890; Cygnus Technologies; Delaware Water Gap, PA; 11 kHz sampling rate per channel) as well as acquired by a custom data acquisition and analysis system. Neurons had to satisfy four criteria to be considered "healthy" and included in the statistical analysis: (a) resting potentials (V_{rest}) had to be -65 mV; (b) action potentials had to overshoot by at least $+20$ mV; (c) V_{rest} varied by ≤ 5 mV between control (Ringer's) and experimental (vehicle or drugs; see below) conditions; and (d) action potential amplitudes (V_{AP}) varied by ≤ 10 mV between control and experimental conditions. Because many of the neurons satisfied all of these criteria, we assumed that exclusion of some neurons was largely due to minor injury from re-impalement or long-duration recordings (see above).

In the conventional current clamp mode, action potentials were elicited by injection of short depolarizing current pulses (1–10 ms, 10 nA). With the data acquisition and analysis system, resting membrane potential (V_{rest}), action potential amplitudes (V_{AP}), action potential half-amplitude durations (D_{AP}), and the maximum rising (dV/dt_{rise}) and falling (dV/dt_{fall}) slopes of action potentials were measured from single sweeps. Averaged sweeps (>10 sweeps) were used to determine the amplitudes of various afterpotential components relative to V_{rest} (see McClellan et al., 2008): V_{fAHP} ; V_{ADP} ; and V_{sAHP} . In the DCC mode, depolarizing

current pulses (2 s, 1–10 nA) were applied to examine repetitive firing patterns. In addition, relatively small 500 ms hyperpolarizing current pulses (ΔI_m) were applied, and the resulting membrane potential deflections ($\Delta V_m < 2$ mV) were used to calculate membrane input resistances ($R_{in} = \Delta V_m / \Delta I_m$) from averaged voltage sweeps.

2.4.3 Application of agents affecting second messenger signaling—With Ringer's solution in the recording chamber, several large, identified RS neurons (2–6 cells) were impaled sequentially, and control electrical recordings were acquired for each cell. Following the last control recording, the last neuron in the sequence often remained impaled during vehicle or drug plus vehicle application to the bath (see below). In other cases, micropipettes were withdrawn while vehicle or drugs were applied, and subsequently neurons were re-impaled. For dbcAMP and dbcGMP (Sigma), 10X concentration solutions in Ringer's were made and added to the recording chamber to obtain final bath concentrations of 10 mM. Forskolin was made up at 250X concentration in ethanol and added to the recording chamber to obtain a final bath concentration of 50 μ M (0.4% ethanol). Approximately 15 min after drug application, experimental recordings began and continued for up to ~4 hrs.

2.4.4 Statistical analysis—First, for control recordings, electrical properties were measured before and after application of vehicle to the bath: Ringer's solution ($n = 11$ uninjured neurons; vehicle for dbcAMP and dbcGMP); 0.4% ethanol in Ringer's solution ($n = 6$ uninjured neurons; vehicle for forskolin); or 0.4% ethanol in Ringer's solution ($n = 7$ injured neurons). For each of these three control conditions, before/after ratios were calculated for each electrical property ("vehicle ratios"). Second, in other experiments, electrical properties were measured before and after application of drug plus vehicle: dbcAMP ($n = 9$ uninjured neurons); forskolin ($n = 18$ uninjured neurons); forskolin ($n = 17$ injured neurons); and dbcGMP ($n = 9$ uninjured neurons). For these four experimental conditions, before and after ratios were calculated for each electrical property ("drug ratios"). Third, in theory it is possible for a vehicle to result in, for example, a non-significant decrease in an electrical parameter and a drug/vehicle to cause a non-significant increase in the parameter, but the difference between vehicle and drug/vehicle conditions might be significant. Therefore, to determine the effects of a given drug on an electrical property, the vehicle ratio values for that property were compared to the corresponding drug ratio values with an unpaired t-test, and with the Welch correction when appropriate. Statistical significance was assumed for $p < 0.05$. Fourth, for plotting the electrical properties in Figures 6 and 10A, each mean and SD for the drug ratios was normalized by the corresponding mean vehicle ratio (e.g. $[r]V_{AP}$; normalized ratios).

If the ADP and sAHP were clearly absent following the main depolarizing phase of action potentials, they were assigned a value of zero. In other cases when it was unclear if particular afterpotential components were present or when they could not be measured (peak of the fAHP depolarized relative to V_{rest} , peak of the ADP hyperpolarized relative to V_{rest}), values were omitted from the ratio analysis described above. Thus, in the summary bar graph (Fig. 7B), there are different n values for different afterpotential components. For the correlation analysis of electrical properties for uninjured RS neurons, voltage shifts of the peaks of the various afterpotential components were plotted against D_{AP} ratios (Fig. 9, 11B). Shifts ($\Delta fAHP$, ΔADP , or $\Delta sAHP$) in the depolarizing direction were given a positive value, while shifts in the hyperpolarizing direction were negative. For injured RS neurons at 2–3 wk recovery times, the ADP and sAHP usually are absent (McClellan et al., 2008), and were not included in the ratio analysis (Fig. 11A) or correlation analysis (Fig. 11B).

3. RESULTS

3.1 Lamprey RS Neurons in Culture

During the pre-control period (2 hrs), DiI-labeled descending brain neurons, of which ~80% are unidentified RS neurons, had neurites that were extending (Fig. 2A) or retracting as growth cones explored their environment. These control growth rates varied from -16.4 to $+19.2$ $\mu\text{m/hr}$. In the present study, most neurons in culture had one (79% of neurons) or two (20%) neurites (Ryan et al., 2007) whose lengths usually were 2–4 times the diameters of the parent cell bodies (Fig. 2B). For 63% of the neurons with two neurites, both neurites were extending or retracting during the pre-control period.

3.2 Second Messenger Effects on Neurite Outgrowth of Lamprey RS Neurons

3.2.1 cAMP signaling pathways—Three different agents were used to activate cAMP pathways and to test their effects on neurite outgrowth of lamprey RS neurons in culture, which allows manipulations not easily performed *in vivo*. First, application of 10 mM dbcAMP, a membrane permeant analogue of cAMP (see Experimental procedures), resulted in a significant increase ($p = 0.001$, $n = 17$ neurites) in neurite outgrowth rates ($\mu\text{m/hr}$) for lamprey RS neurons whose processes were retracting during the pre-control period (see Experimental procedures) (Fig. 3A, left pair of bars). In contrast, for neurites that were extending during the pre-control period, dbcAMP did not have a significant effect on outgrowth rates ($n = 23$; Fig. 3A, right pair of bars). In control experiments, addition to the bath of comparable volumes of Ringer's solution, the vehicle for dbcAMP, did not significantly alter neurite outgrowth rates (data not shown; see Ryan et al., 2007). In summary, the actions of dbcAMP on neurite outgrowth are dependent on initial growth state (see Discussion).

Second, application of 100 μM forskolin, which activates adenylyl cyclase and stimulates the synthesis of cAMP (see Experimental procedures), also resulted in a significant increase in neurite outgrowth rates for neurons whose processes were retracting during the pre-control period ($p = 0.0001$, $n = 24$; Fig. 3B, left pair of bars). However, for neurons with extending processes during the pre-control period, forskolin did not significantly affect neurite outgrowth rates ($n = 20$; Fig. 3B, right pair of bars), similar to the results for dbcAMP. In control experiments, 0.4% DMSO, the vehicle for forskolin, did not significantly alter neurite outgrowth rates (data not shown).

Third, application of 100 μM IBMX, a phosphodiesterase inhibitor that blocks degradation of endogenous cAMP, to RS neurons with neurites that initially were retracting during the pre-control period resulted in a significant increase in neurite outgrowth rates ($p = 0.001$, $n = 31$; Fig. 3C, left pair of bars). Thus, during the pre-control period, these neurons in culture presumably were synthesizing cAMP, but not at sufficient levels to mediate neurite outgrowth. In contrast, for neurons that were extending their neurites during the pre-control period, IBMX did not significantly alter neurite outgrowth rates ($n = 22$; Fig. 3C, right pair of bars). Therefore, the response to IBMX was growth state-dependent, similar to the results for both dbcAMP and forskolin.

Fourth, for neurons with two neurites that both were retracting during the pre-control period, dbcAMP, forskolin, and IBMX consistently resulted in a switch to extension for both neurites in 10/11 neurons (see column R $\rightarrow$ E in Table 1). In contrast, for neurons in which both neurites initially were extending, activation of cAMP signaling resulted in a switch to retraction for both neurites in 3/7 neurons (see column E $\rightarrow$ R in Table 1).

3.2.2 Involvement of PKA in cAMP-mediated neurite stimulation—One of the signaling pathways activated by cAMP involves PKA. To test whether neurite outgrowth of lamprey RS neurons in culture depends on PKA, the effects of three different PKA inhibitors were tested. For RS neurons that were extending their neurites during the pre-control periods, application of 10 μ M H89 ($n = 9$, Fig. 4A; right pair of bars), 10 μ M 1NM-PP1 ($n = 19$, Fig. 4B), or 30 μ M PKI(14–22) ($n = 17$, Fig. 4C) usually resulted in neurite retraction and a significant decrease in neurite outgrowth rates. Unlike PKI(14–22) (Dalton et al., 2005), H89 and 1NM-PP1 are not specific PKA inhibitors (Davies et al., 2000; Bain et al., 2007), but the similar effects of all three agents suggest that the main mechanism of action here was inhibition of PKA. These results provide additional evidence suggesting that RS neurons in culture were synthesizing cAMP, and blocking PKA downstream negated the growth promoting actions of this second messenger. In contrast, for neurons with retracting neurites during pre-control periods, inhibiting PKA with H89 ($n = 10$), 1NM-PP1 ($n = 29$), or PKI(14–22) ($n = 11$) did not significantly alter outgrowth rates (Fig. 4A,B,C; left pairs of bars). Thus, these PKA inhibitors affect neurite outgrowth in a growth state-dependent fashion.

For neurons with two neurites that both were extending during the pre-control period, H-89, 1NM-PP1, and PKI(14–22) converted both neurites to retraction for 3/5 neurons (see column E $\rightarrow$ R in Table 1). In contrast, for neurons with two neurites that both initially were retracting, inhibition of PKA signaling was ineffective in switching both neurites to extension for 0/5 neurons (see column R $\rightarrow$ E in Table 1).

3.2.3 cGMP signaling pathways—Application of 10 mM dbcGMP, a membrane permeant analogue of cGMP, resulted in a significant decrease in neurite outgrowth rates for lamprey RS neurons that were extending neurites during the pre-control period ($p = 0.0001$, $n = 29$; Fig. 5, right pair of bars). However, no significant effects of this agent were found for neurons whose neurites were retracting during the pre-control period ($n = 26$, Fig. 5 left pair of bars). Thus, similar to the agents above that activated cAMP signaling or inhibited PKA, the effects of activation of cGMP signaling also were dependent on the initial growth state of neurites. Because activation of cGMP signaling inhibited neurite outgrowth of lamprey RS neurons in culture, the specific downstream signaling pathways (e.g. PKG) were not explored further.

For neurons with two neurites that both were extending during the pre-control period, dbcGMP resulted in a switch to retraction for both neurites in 3/4 neurons (column E $\rightarrow$ R in Table 1). In contrast, for neurons in which both neurites initially were retracting, activation of cGMP signaling only switched 1/4 pairs of neurites to extension (see column R $\rightarrow$ E in Table 1).

3.2.4 Temporal effects of agents—For some neurites, application of the drugs described above seemed to result in effects within 10–20 min. However, in some cases the effects of the drugs were variable over this short time course, and therefore, growth rates were determined over a 120 min experimental period, during which the drug effects were much more consistent and robust.

3.3 Effects of Second Messenger Signaling on Properties of Uninjured RS Neurons

3.3.1 cAMP signaling pathways—The effects of cAMP signaling on electrical properties of lamprey RS neurons were tested in isolated brain-spinal cord preparations (Fig. 6A; see Experimental procedures), which allowed a comparison of drug effects on uninjured and injured neurons. Two different agents were used to activate cAMP signaling in uninjured RS neurons. First, application of 10 mM dbcAMP did not significantly change the

normalized ratios (see Experimental procedures) for any of the electrical properties of uninjured RS neurons (Fig. 6B,7). In particular, dbcAMP did not cause spike broadening and/or an increase in the sAHP, which potentially might indicate an increase in calcium influx.

Second, in contrast to dbcAMP, application of 50 μ M forskolin to uninjured RS neurons resulted in a significant increase (mean = +40%) in the normalized ratio for action potential duration ($[r]D_{AP}$; see Experimental procedures) ($p < 0.01$, $n = 18$ neurons) (Figs. 6C1,7A). This increase in duration appeared to be mediated, in part, by a significant reduction in the normalized ratios for the slopes of the rising phase (mean $[r]dV/dt_{rise} = -23\%$) and falling phase (mean $[r]dV/dt_{fall} = -30\%$) of action potentials ($p < 0.01$, $n = 18$, Fig. 7A). These decreases in both the rising and the falling slopes suggest that forskolin affected both Na^+ and K^+ voltage-gated channels. Activation of cAMP signaling with forskolin did not significantly change the normalized ratio for input resistance ($[r]R_{in}$; $p = 0.05$, $n = 18$, Fig. 7A). Also, forskolin did not significantly affect the normalized resting membrane potential ratio ($[r]V_{rest}$), action potential amplitude ratio ($[r]V_{AP}$) (Figs. 6C1,7A), or the ratios for the amplitudes of the ADP or sAHP ($[r]V_{ADP}$, $[r]V_{sAHP}$) (Figs. 6C2,7B). The normalized ratio for the fAHP was significantly reduced by -64% ($p < 0.01$, $n = 14$, Fig. 7A). Because dbcAMP should have very similar actions as endogenous cAMP, these results suggest that forskolin may have had some effects in addition to activation of cAMP signaling pathways (see Discussion).

3.3.2 cGMP signaling pathways—Application of 10 mM dbcGMP did not significantly change any of the normalized ratios for electrical properties of uninjured RS neurons (Fig. 7). Thus, although dbcAMP and dbcGMP had opposite effects on neurite outgrowth of RS neurons in culture, neither drug appeared to significantly affect the electrical properties of these neurons.

3.4 Second Messenger Signaling did not Alter Firing of Uninjured RS Neurons

Prior to drug application, uninjured RS neurons fired a smooth train of action potentials with moderate spike frequency adaptation in response to applied 2 s depolarizing current pulses (see Experimental procedures) (Fig. 8B1-D1). In the presence of 10 mM dbcAMP, 50 μ M forskolin, or 10 mM dbcGMP, RS neurons did not appear to substantially alter their firing patterns (Fig. 8B2-D2). For all three drugs, the half-amplitude durations of the sAHP were measured, when present, and the average normalized ratios for $[r]D_{sAHP}$ were not significantly different than 1.0. Thus, the lack of effect of the drugs on the duration of the sAHP probably explains, in part, why the firing patterns were not noticeably affected (see Meer and Buchanan. 1992; El Manira et al., 1994).

3.5 Additional Analysis of Effects of Forskolin on Uninjured RS Neurons

Forskolin increased the normalized ratio for action potential duration ($[r]D_{AP}$) of lamprey RS neurons (Fig. 6,7A), and this effect potentially might enhance calcium influx (McCobb and Beam, 1991; Toth and Miller, 1995) and possibly compromise the growth promoting actions of this agent (see Introduction). Therefore, the effects of forskolin on uninjured RS neurons were further investigated.

3.5.1 Forskolin shifted the peaks of the fAHP and ADP but not sAHP—In some cases for uninjured RS neurons, the control amplitudes of the afterpotential components, particularly the ADP, were relatively small (i.e. peaks close to V_{rest}), and this could bias the ratio analysis described above (see $[r]V_{ADP}$ in Fig. 7B, see Experimental procedures). Consequently, a correlation analysis was performed to examine the effects of forskolin on voltage shifts of the peaks of the fAHP, ADP and sAHP vs. the ratios for action potential

duration (D_{AP} ratio). In the presence of 50 μ M forskolin, there was a significant correlation of the shifts in the peaks of the fAHP ($\Delta fAHP$) vs. D_{AP} ratios (Fig. 9A, $p = 0.01$) as well as the ADP (ΔADP) vs D_{AP} ratios ($p = 0.05$, Fig. 9B). In contrast, forskolin did not result in a significant correlation for shifts in the peaks of the sAHP ($\Delta sAHP$) vs D_{AP} ratios (Fig. 9C). Taken together, the data suggest that in response to forskolin, spike broadening and increases in the duration of action potentials (D_{AP}) had a temporal cascade effect and tended to shift the peaks of the fAHP and, to a slightly lesser extent, the ADP in a depolarizing direction, but had little effect on the peak of the sAHP.

3.6 Forskolin did not Substantially Alter Electrical Properties of Injured RS Neurons

For the various agents that were tested on electrical properties of uninjured lamprey RS neurons, forskolin was the only drug that increased action potential duration, which very likely would increase calcium influx and might potentially compromise its growth promoting actions. Therefore, the effects of forskolin were further investigated for injured RS neurons during the time they were regenerating their descending axons.

At short recovery times (2–3 wks) following spinal cord injury (see Experimental procedures, Fig. 10A), axotomy-induced changes in neurophysiological properties of lamprey RS neurons are most pronounced (McClellan et al., 2002; see Experimental procedures). In addition, the fAHP is significantly larger than those in uninjured neurons, and the ADP and sAHP are absent or significantly reduced (McClellan et al., 2008). In contrast to uninjured RS neurons, for injured RS neurons 50 μ M forskolin did not significantly affect any of the measured electrical properties (Figs. 10B1,11A). Specifically, forskolin did not significantly alter the normalized ratio for the action potential durations ($[r]D_{AP}$) or the normalized ratio for the amplitudes of the fAHP ($[r]V_{fAHP}$) (Fig. 11A). Moreover, unlike the situation for uninjured lamprey RS neurons (Fig. 9A), for injured neurons, forskolin did not result in a significant correlation between the voltage shifts of the peaks of the fAHP ($\Delta fAHP$) and the D_{AP} ratios (Fig. 11B).

3.6.1 Effects of Forskolin on firing patterns of injured RS neurons—Compared to uninjured RS neurons (Fig. 8B1-D1), axotomized neurons at 2–3 wks following spinal cord injury display substantial changes in their firing patterns, as previously described (McClellan et al., 2008). Specifically, in the present study, in response to applied 2 s depolarizing current pulses, injured neurons fired a single short burst (Fig. 10C1) or short, repetitive bursts of action potentials (not shown). Compared to control recordings, application of 50 μ M forskolin resulted in a significantly more depolarized voltage threshold (11 of 12 injured RS neurons; $p = 0.006$, Sign test), and fewer action potentials for a given applied suprathreshold 2 s depolarizing current pulse (Fig. 10C1,C2).

4. DISCUSSION

4.1 Activation of Second Messengers Regulates Neurite Outgrowth

4.1.1 cAMP signaling pathways—The present results suggest that activation of cAMP pathways conditionally stimulates neurite outgrowth of lamprey RS neurons in culture, which are presumed to be unidentified neurons (see Experimental Procedures), dependent on initial growth state (Fig. 3). For neurons that initially were retracting their processes during the pre-control period, cAMP pathways might have been activated at a low level, and stimulation of these pathways then caused a switch to a neurite outgrowth mode. Likewise, for neurons that initially were extending processes, cAMP pathways might have already been activated near maximal, and additional activation of these pathways was largely ineffective. Further support for this notion comes from experiments with PKA inhibitors (H89, 1NM-PP1, PKI(14–22) (Fig. 4). For example, for neurons that initially were

extending their processes and presumably synthesizing cAMP, inhibition of PKA converted neurons to a retracting mode, but had little effect on neurites that initially were already retracting and that might have had low levels of cAMP. Taken together, these results suggest that for cultured lamprey RS neurons, activation of cAMP signaling stimulates neurite outgrowth depending on initial growth state and, at least in part, in a PKA-dependent manner.

In spinal cord-injured larval lampreys, fluorescently labeled Müller cells (large, identified RS neurons) have been imaged at early recovery times when the axon tips of these neurons were rostral to the spinal injury site (Jin et al., 2009). The axon tips of these large neurons were relatively simple and often bulb-shaped. Repeated exposure and imaging of the spinal cord over 2–48 hours suggested that axonal extension (regeneration) was intermittent or discontinuous. However, with this repeated imaging approach, axonal regeneration was never observed in real time. Activation of cAMP signaling with dbcAMP enhanced axonal regeneration of these neurons by increasing the velocity of axonal extension but not the fraction of time spent in an extending mode.

In numerous studies using different neuronal types from a variety of animals, activation of cAMP signaling often stimulates neurite outgrowth in culture (Filbin, 2003), often via PKA-dependent mechanisms (Cai et al., 2001). For example, either dbcAMP or IBMX enhances neurite outgrowth of chick olfactory neurons (Johnson et al., 1988). Moreover, inhibition of phosphodiesterases (PDEs), which degrade endogenous cAMP, promotes neurite outgrowth of rat DRG neurons (Nikulina et al., 2004). Also, application of forskolin to cultured rat dorsal root ganglion (DRG) neurons (Neumann et al., 2002) or spiral ganglion neurons (Xu et al., 2012) stimulates neurite outgrowth. In contrast, forskolin and dbcAMP both suppress neurite elongation for *Helisoma* neurons in culture (Mattson et al., 1988).

Various agents that activate cAMP signaling pathways can stimulate axonal regeneration following injury (Qui et al., 2002). In rats with spinal cord injuries, injection of dbcAMP into DRGs promotes regeneration of sensory axons in the dorsal columns (Neumann et al., 2002). Also, in spinal cord-lesioned zebrafish, application of dbcAMP improves axonal regeneration of Mauthner cells (Bhatt et al., 2004), which normally regenerate poorly following SCI. Finally, for injured frog peripheral nerves, forskolin activates the synthesis of cAMP and stimulates sensory axon regeneration (Kilmer and Carlsen, 1984).

4.1.2 cGMP signaling pathways—In contrast to the effects of cAMP signaling, activation of cGMP pathways in lamprey RS neurons in culture inhibited neurite outgrowth depending on initial growth state (Fig. 5). For neurons whose neurites initially were extending processes, cGMP levels might have been low, while in neurons that initially were retracting, cGMP signaling pathways might have already been activated near maximal. Because activation of cGMP signaling inhibited neurite outgrowth of lamprey RS neurons, the specific down-stream signaling pathways were not explored further.

For cultured neurons in other systems, activation of cGMP signaling results in variable effects on neurite outgrowth. For example, high levels of cGMP relative to cAMP lead to growth cone repulsion of *Xenopus* spinal neurons, while the opposite leads to attraction (Nishiyama et al., 2003). Moreover, for rat embryonic hippocampal neurons, activation of cGMP signaling suppresses axon formation but specifically promotes dendritic outgrowth (Shelly et al., 2010). In contrast, activation of cGMP signaling promotes neurite outgrowth of rat DRG neurons (Murray et al., 2009) and neurons in adult goldfish retinal explants (Koriyama et al., 2009). Interestingly, *in vivo* cGMP signaling stimulates regeneration of retinal axons in the injured goldfish optic nerve (Koriyama et al., 2009) and axons in the injured locust ventral nerve cord (Stern and Bricker, 2010).

4.2 Effects of Second Messenger Signaling on the Electrical Properties of Uninjured RS Neurons

4.2.1 cAMP signaling pathways—For uninjured RS neurons, dbcAMP did not significantly alter any of the measured electrical properties (Fig. 6B,6). In contrast, forskolin significantly increased the normalized ratio for action potential duration ($[r]D_{AP}$), due in part to a decrease in the rising and falling slopes of spikes (Figs. 6C1,7A), suggesting that both Na^+ and K^+ voltage-gated channels might be affected. Because forskolin appeared to alter voltage-gated ion channels of lamprey RS neurons, it is unlikely this agent mediated its effects via cyclic nucleotide-gated channels (CNG), which are virtually voltage independent (Craven and Zagotta 2006). In addition, action potential broadening was correlated with a significant depolarizing shift of the peaks of the fAHP and ADP (Fig. 9A,B) but not the sAHP (Fig. 9C). Thus, these shifts in the peaks of the fAHP and ADP appear to be an indirect effect of action potential broadening. Furthermore, neither forskolin nor dbcAMP appeared to modify the firing patterns of uninjured RS neurons, which suggests that the underlying key electrical properties that affect firing, such as the sAHP (see Meer and Buchanan. 1992; El Manira et al., 1994), were not substantially modified.

In some other studies, forskolin has effects on neuronal electrical properties that are comparable to those in the present study. For different types of neurons in multiple systems, forskolin often blocks or reduces voltage-gated K^+ currents (Grega et al., 1987; Harris-Warrick 1989; Herness et al., 1997), and in embryonic chick sensory neurons this effect results in action potential broadening (Dunlap, 1985). For lamprey sensory neurons, forskolin does not appear to substantially affect sodium-based action potentials (Womble and Wickelgren 1990; however see Parker et al., 1997) but does broaden Ca^{2+} action potentials by inhibiting K_{Ca} channels (Womble and Wickelgren, 1989; also see Grega and MacDonald, 1987). However, in the present study, the sAHP, which is mediated, in part, by K_{Ca} channels (McClellan et al., 2008), was not affected significantly by forskolin (Fig. 6C2,7B).

Although forskolin and dbcAMP have some similar actions on the electrical properties of neurons (see Grega and MacDonald, 1987; Tokimasa and Akasu, 1990), they also have distinct effects (see Watanabe and Gola, 1987; Harris-Warrick, 1989; Herness et al., 1997). For example, in lamprey sensory neurons, forskolin, but not dbcAMP, increases the duration of Ca^{2+} action potentials (Leonard and Wickelgren 1986). Thus, in the present study, forskolin might have affected electrical properties, in part, via cAMP- and PKA-independent mechanisms. Consequently, the above data from other studies might explain some of the different effects dbcAMP and forskolin had on the electrical properties of lamprey RS neurons (Figs. 7). Because dbcAMP stimulated neurite outgrowth without altering the electrical properties of lamprey RS neurons, this agent seems to be well suited for stimulating axonal regeneration following SCI. In contrast, forskolin resulted in action potential broadening and very likely would increase calcium influx (McCobb and Beam, 1991; Toth and Miller, 1995), which is known to compromise neurite outgrowth in culture (Kater, 1991; Bandtlow et al., 1993; Moorman and Hume 1993; Ryan et al., 2007) as well as axonal regeneration following neuronal injury (Enes et al., 2010).

4.2.2 cGMP signaling pathways—Application of dbcGMP did not result in significant changes in the electrical properties of uninjured lamprey RS neurons (Figs. 6D,7). Interestingly, for lamprey sensory neurons, dbcGMP does not alter the duration of Ca^{2+} action potentials (Leonard and Wickelgren 1986).

For neurons in other systems, activation of cGMP pathways has variable effects on electrical properties. For example, in human neuroblastoma cells, activation of cGMP signaling reduces voltage-gated calcium currents in a PKG-dependent fashion (D'Ascenzo et al.,

2002). However, for guinea pig hippocampus neurons, the cGMP-mediated decrease in calcium currents is PKG-independent (Doerner and Alger, 1988). For newt olfactory receptor cells, activation of cGMP pathways increases both calcium and sodium currents, but has no significant effects on potassium currents (Kawai and Miyachi 2001).

4.3 Effects of Forskolin on Properties of Injured RS Neurons

For injured lamprey RS neurons, forskolin did not result in changes in any of the measured action potential parameters, including the normalized ratios for action potential duration ($[r]D_{AP}$) or the fAHP amplitude ($[r]V_{fAHP}$) (Figs. 10B1,B2,11A). Moreover, forskolin did not affect the input resistance or substantially alter the firing patterns of these neurons (Fig. 10C1,C2). Thus, for injured lamprey RS neurons, forskolin did not increase action potential durations or repetitive firing rates, which would be expected to increase calcium influx (McCobb and Beam, 1991; Toth and Miller, 1995; Ross 1989) and potentially compromise the growth promoting effects of this agent (Ryan et al., 2007; McClellan et al., 2008).

4.4 Conclusions

For lamprey RS neurons in culture, activation of cAMP pathways stimulated neurite outgrowth dependent on initial growth state and in a PKA-mediated manner. In contrast, stimulation of cGMP pathways inhibited outgrowth, also dependent on initial growth state. Forskolin resulted in action potential broadening, at least for uninjured RS neurons, and this effect would very likely increase calcium influx (McCobb and Beam, 1991; Toth and Miller, 1995), which is known to compromise neurite outgrowth in culture as well as axonal regeneration following neuronal injury. In contrast, for lamprey RS neurons, dbcAMP stimulated neurite outgrowth without altering their electrical properties in such a way that would be expected to increase calcium influx, and therefore this agent seems to be well suited for stimulating axonal regeneration following SCI. In conclusion, results from the present study suggest that activation of cAMP signaling alone, without compensation for possible deleterious effects on electrical properties, is an effective approach for stimulating axonal regeneration of RS neurons following spinal cord injury.

Acknowledgments

We thank Mirela Milesescu, David Schulz, and Lorin Milesescu for comments on an earlier version of this manuscript. We are grateful to Carl Groat for excellent technical assistance, and Taylor Pancoast, Sean Bennett, and Leigh Rettenmaier for help with analysis of the data. Supported by NIH grant NS 29043, University of Missouri (UM) Spinal Cord Injury Program, UM Research Board, and Research Council awarded to A.D.M., and Sigma XI predoctoral grant to T.P.

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Highlights

- cAMP stimulated neurite outgrowth of lamprey RS neurons in culture
- blockers of PKA inhibited neurite outgrowth of lamprey RS neurons
- forskolin, but not dbcAMP, altered the electrical properties of lamprey RS neurons
- dbcAMP is a fully effective method to stimulate axonal regeneration of RS neurons

Figure 1.

(upper) Diagram of dorsal view of a larval lamprey brain (left) and rostral spinal cord showing the transections (dotted lines) where the brain was cut into blocks for neuronal cell culture (see Experimental procedures). Contours represent cell groups containing descending brain neurons that project to the spinal cord (see Davis and McClellan, 1994a,b). Reticulospinal (RS) neurons, which account for 80% of descending brain neurons, are located in the mesencephalic reticular nucleus (MRN) and the anterior (ARRN), middle (MRRN) and posterior (PRRN) rhombencephalic reticular nuclei. Other descending brain neurons are located in the anterolateral (ALV), dorsolateral (DLV), and posterolateral (PLV) vagal groups. (lower) Enlargement of reticular nuclei showing large, uniquely identifiable RS neurons (Müller cells) in the MRN (M1–M3), ARRN (I1–I4), and MRRN (B1–B5). The Mauthner (Mau) and auxiliary Mauthner (AM) cells are located in the MRRN. Unidentified neurons are omitted for simplicity.

Figure 2.

(A) Sequential images (A1–A4), each separated by ~36 min, showing a DiI-labeled descending brain neuron (arrows indicate cell body) whose associated neurite, which terminated in a growth cone (arrowheads), extended during the Pre-Control period (compare arrowhead and dotted line in A4). Scale bar = 50 μ m. (B) Histogram displaying the relative distributions of the initial neurite length-to-cell body diameter ratios for all neurons from the Pre-Control periods for dbcAMP, forskolin, and IBMX ($n = 139$ neurites). For most neurons in culture, the neurite length was 2–4 times the cell body diameter.

Figure 3.

Activation of cAMP pathways stimulated neurite outgrowth of lamprey descending brain neurons, 80% of which are RS neurons. Average neurite outgrowth rates (bars = means, vertical lines = SDs) prior to addition of agents (Pre-Control, open bars, 120 min), and during the presence of (A) 10 mM dbcAMP, (B) 100 μM forskolin, or (C) 100 μM IBMX (Experimental, black filled bars, 120 min). Outgrowth rates for neurites that were retracting (left pairs of bars) or extending (right pairs of bars) during the Pre-Control period. The ratios beside each filled bar indicate the fraction of neurites that responded to a given agent in the same way (extension or retraction) as the overall average effect. Statistics: paired t-test for

neurite outgrowth rates between Pre-Control and dbcAMP, forskolin, or IBMX; NS = not significantly different.

Figure 4.

Blocking PKA inhibited neurite outgrowth. Average neurite outgrowth rates (bars = means, vertical lines = SDs) prior to addition of agents (Pre-Control, open bars, 120 min), and during the presence of (A) 10 μ M H89, (B) 10 μ M 1NM-PP1, or (C) 30 μ M PKI(14-22) (Experimental, black bars, 120 min); NS = not significantly different. See Fig. 3 legend for additional explanation.

Figure 5.

Activation of cGMP inhibited neurite outgrowth. Average neurite outgrowth rates (bars = means, vertical lines = SDs) prior to addition of dbcGMP (Pre-Control, open bars, 120 min), and in the presence of 10 mM dbcGMP (Experimental, black bars, 120 min); NS = not significantly different. See Fig. 3 legend for additional explanation.

Figure 6.

The agents dbcAMP or dbcGMP, unlike forskolin, did not alter action potential properties of uninjured, identified RS neurons (also see Fig. 7). (A) Isolated brain-spinal cord preparation showing the brain (left), and rostral spinal cord, intracellular recording micropipette (IC), and suction electrode around the caudal end of the spinal cord (SC) (see Experimental procedures). (B) (B1) Action potentials from a right uninjured "I1 cell" (see Fig. 1) for control conditions and in the presence of 10 mM dbcAMP. (B2) Control and dbcAMP traces from the same neuron as in panel B1 showing afterpotentials with three components: fast after hyperpolarization (fAHP); after depolarization (ADP); and slow AHP (sAHP). (C) (C1) Action potentials for a right uninjured "B3 cell" (see Fig. 1) under control conditions

and in the presence of 50 M forskolin. (C2) Control and forskolin recordings from the same neuron as in panel C1 showing afterpotential components. (D) (D1) Action potentials (control and 10 mM dbcGMP) from a right uninjured “B4 cell”. (D2) Control and dbcGMP traces from the same neuron as in panel D1 showing afterpotential components. For B1, C1 and D1, the x,y coordinates of the raw action potential traces were imported into Excel (Microsoft; Seattle, WA) and plotted with the smoothing line tool to extrapolate between the sample points, which were acquired at 10 kHz. Scale Bars: (vertical/horizontal) 30 mV/1 ms (B1, C1, D1); 3 mV/50 ms (B2, C2, D2).

Figure 7.

Normalized ratio values (see Experimental procedures) for various components of action potentials and passive membrane properties of uninjured, identified RS neurons before and after application of 10 mM dbcAMP (gray bars), 50 μ M forskolin (open bars), or 10 mM dbcGMP (black bars). Normalized ratio values = drug ratios / vehicle ratios. Bars = means, vertical lines = SDs. Statistics: unpaired t-test (for each parameter, drug ratio values were compared to vehicle ratio values; see Experimental procedures) *- p < 0.05, ** - p < 0.01, *** - p < 0.001.

Figure 8.

Activation of second messenger signaling pathways with dbcAMP, forskolin, or dbcGMP did not appear to alter firing patterns of uninjured RS neurons. (A) Isolated brain-spinal cord preparation (see legend for Fig. 6A). (B,C,D) Membrane potential (V), current (I), and instantaneous firing frequency (F). (B) Firing patterns for a right "I1 cell" in response to a 2 s depolarizing current pulse before (B1) and after (B2) application of 10 mM dbcAMP. (C) Firing pattern of left "B3 cell" before (C1) and after (C2) application of 50 μ M forskolin. (D) Firing patterns of a different right "I1 cell" before (D1) and after (D2) application of 10 mM dbcGMP. Scale bars: (vertical) 75 mV/6 nA/5 Hz / (horizontal) 500 ms.

Figure 9.

Correlation analysis of voltage shifts (Δ) of the peaks of the various afterpotential components vs. the D_{AP} ratios (forskolin / Ringer's) for uninjured RS neurons. (A) Δ fAHP ($n = 14$ neurons), (B) Δ ADP ($n = 11$), and (C) Δ sAHP ($n = 16$) vs. D_{AP} ratios. In the presence of forskolin, larger increases in the D_{AP} ratios were correlated with depolarizing shifts in the peaks of the fAHP and ADP, but with little effect on the peaks of the sAHP.

Figure 10.

Activation of cAMP signaling in injured RS neurons did not substantially alter action potential properties and firing patterns. (A) Isolated brain-spinal cord preparation showing the brain (left), and rostral spinal cord, double hemi-transections (HTs; see Experimental procedures) at 10% BL (2–3 wks post-lesion), intracellular recording micropipette (IC), suction electrode on the dorsal surface of the spinal cord above the HTs (SC1), and suction electrode around the caudal end of the spinal cord below the HTs (SC2) (see Experimental procedures). (B) (B1) Action potential traces from a left injured “B3 cell” before and after application of 50 μ M forskolin. (B2) Superimposed control and forskolin traces from the same neuron as in panel B1, showing only the fAHP. (C) Membrane potential (V), current

(I), and firing frequency (F). Firing of an injured left “B3 cell” (same cell as in B) in response to a 2 s depolarizing current pulse before (C1) and after (C2) application of 50 μ M forskolin. Scale bars: (vertical/horizontal) (B1) 30 mV/2 ms, (B2) 5 mV/95 ms. (C1, C2) (vertical) 20 mV/10 nA/30 Hz / (horizontal) 1 s.

Figure 11.

(A) Summary of action potential components and passive membrane properties of injured, identified RS neurons before and after application of 50 μ M forskolin. Normalized ratio values = drug ratios / vehicle ratios (see Experimental procedures). Bars = means, vertical lines = SDs. Statistics: unpaired t-test (for each parameter, the drug ratios were compared to vehicle ratios; see Experimental procedures). There were no significant changes in response to forskolin. Note that the ADP and sAHP often are absent in injured lamprey RS neurons. (B) In contrast to uninjured RS neurons (Fig. 9), for injured, identified RS neurons ($n = 17$ neurons), forskolin did not result in voltage shifts in the peaks of the fAHP (Δ fAHP) that were correlated with the D_{AP} ratios.

TABLE 1

Response of Neurons with Two Neurites to Bath-Applied Agents

	Total^a	R → E^b	E → R^c	E-R^d
dbcAMP	7	3/3	0/2	2
Forskolin	7	4/5	1/1	1
IBMX	13	3/3	2/4	
H-89	3	0/0	1/2	6
1NM-PP1	10	0/5	1/1	4
PKI(14–22)	3	0/0	1/2	1
dbcGMP	14	1/4	3/4	6

^a total numbers of neurons analyzed with two neurites (total sum = 57 neurons)

^b ratio of number of neurites that both switched to extension from retraction divided by total numbers of neurites that both were retracting during the Pre-Control Period

^c ratio of number of neurites that both switched to retraction from extension divided by total numbers of neurites that both were extending during the Pre-Control Period

^d number of neurons with two neurites in which one neurite was extending and the other neurite was retracting during the Pre-Control Period

cAMP Stimulates Neurite Outgrowth of Lamprey Reticulospinal Neurons without Substantially Altering Their Biophysical Properties