Neuron, Vol. 35, 711–719, August 15, 2002, Copyright ©2002 by Cell Press

Arginase I and Polyamines Act Downstream from Cyclic AMP in Overcoming Inhibition of Axonal Growth MAG and Myelin In Vitro

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Summary

Elevation of cAMP can overcome myelin inhibitors to encourage regeneration of the CNS. We show that a consequence of elevated cAMP is the synthesis of polyamines, resulting from an up-regulation of Arginase I, a key enzyme in their synthesis. Inhibiting polyamine synthesis blocks the cAMP effect on regeneration. Either over-expression of Arginase I or exogenous polyamines can overcome inhibition by MAG and by myelin in general. While MAG/myelin support the growth of young DRG neurons, they become inhibitory as DRGs mature. Endogenous Arginase I levels are high in young DRGs but drop spontaneously at an age that coincides with the switch from promotion to inhibition by MAG/myelin. Over-expressing Arginase I in maturing DRGs blocks that switch. Arginase I and polyamines are more specific targets than cAMP for intervention to encourage regeneration after CNS injury.

Introduction

After injury, the adult CNS shows little spontaneous regeneration (Schwab and Bartholdi, 1996). Inhibitors of regeneration present in myelin are major obstacles to axonal regeneration, particularly immediately after injury (Carbonetto et al., 1987; Caroni and Schwab, 1988; Huang et al., 1999). To date, three inhibitors in myelin have been identified: Nogo, an antigen of the IN-1 antibody (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), and recently, myelin-oligodendrocyte glycoprotein (OMgp) (Wang et al., 2002). Interestingly, to exert inhibition, all three of these proteins interact with the same receptor, initially identified as the Nogo receptor (NgR) (Domeniconi et al., 2002, Liu et al., 2002; Wang et al., 2002).

In contrast to the mature CNS, many young neurons do regenerate in vivo (Bates and Stelzner, 1993; Bregman and Goldberger, 1983) and are not inhibited by MAG (DeBellard et al., 1996). Their growth is promoted by MAG, and they can extend long neurites when grown on a substrate of total myelin (Cai et al., 2001). With maturation, all neurons studied so far switch their response to MAG and myelin from promotion to inhibition. The switch occurs predictably at the same age for a particular type of neuron, but the timing of the switch varies according to neuronal type (Cai et al., 2001; De Bellard and Filbin, 1999). Significantly, the switch from promotion to inhibition by MAG and myelin often coincides with the loss of ability to regenerate spontaneously in vivo (Cai et al., 2001). Therefore, the response of a neuron to MAG and myelin in culture is a strong indicator of its capacity to regenerate in vivo.

Recently, we showed that elevating neuronal cAMP, either with a cAMP analog or by exposure to various neurotrophins (NT), overcomes inhibition by MAG and myelin (Cai et al., 1999). Importantly, elevated cAMP in vivo results in regeneration of mature spinal axons (Qiu et al., 2002; Neumann et al., 2002) and plays a role in the spontaneous regeneration of neonatal spinal cord axons after injury (Cai et al., 2001). The downstream consequences of elevated cAMP and activation of protein kinase A (PKA), a cAMP effector that results in regeneration on myelin and in vivo, are not known. It is possible that PKA affects axonal growth through a direct action on cytoskeletal behavior or indirectly by initiating transcription of specific genes.

One candidate gene that is known to be upregulated in response to cAMP in liver (Nebes and Morris, 1988) and macrophages (Morris et al., 1998) is the gene for Arginase I (Arg I), an enzyme shown to have an anti-apoptotic effect in neurons (Esch et al., 1998). Arginase exists in two distinct isoforms, one cytosolic (Arg I) and the second mitochondrial (Arg II) (Nakamura et al., 1990). Both catalyze the hydrolysis of arginine to ornithine and urea. Ornithine is then converted in the cytosol to the polyamine putrescine by the action of ornithine decarboxylase (ODC); putrescine is converted to two other polyamines, spermidine and spermine (Seiler, 2000). Many studies have shown that polyamines influence growth and development of the nervous system (Slotkin and Bartolome, 1986; Slotkin et al., 1982) as well as axonal regeneration in systems that exhibit spontaneous regrowth after injury (Gilad and Gilad, 1988; Gilad et al., 1996; Ingoglia et al., 1982).

Until now, a role for Arg I and its upregulation by cAMP in the nervous system has never been shown. Here, we demonstrate that a transcription-dependent up-regulation of Arg I accompanies the loss of inhibition by MAG and myelin from elevated neuronal cAMP, either via db cAMP or the neurotrophin BDNF (brain-derived nerve growth factor). We also show that direct inhibition of polyamine synthesis blocks the ability of both db cAMP and BDNF to overcome inhibition. Furthermore, overexpression of Arg I and exogenous polyamines are each individually sufficient to overcome inhibition by MAG

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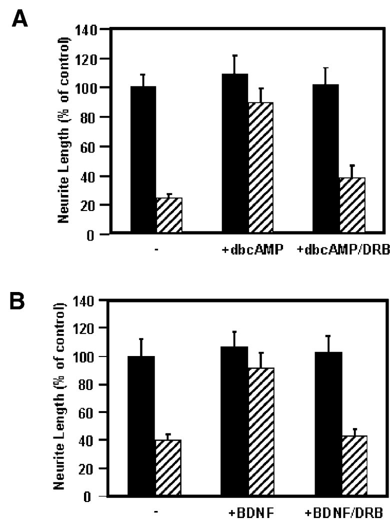

Figure 1. The Ability of db cAMP and BDNF to Overcome Inhibition by MAG Is Transcription Dependent

Cerebellar neurons (P5) were isolated and either added directly to MAG-expressing CHO cells (stripped bars) or control CHO cells (black bars) in the presence of db cAMP (1 mM), with and without DRB (5 μM) as indicated (A) or after being primed overnight with BDNF (200 ng/ml) (B) after which they were cultured overnight, fixed, and immunostained for GAP43. In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM) for at least four separate experiments.

and myelin. Finally, young neurons that grow well on MAG and myelin also show high levels of Arg I while in older neurons, growth is inhibited by MAG and myelin, and Arg I levels have spontaneously decreased. Inhibiting ODC blocks the growth of young neurons on MAG and myelin, and overexpressing Arg I in older neurons abolishes their growth inhibition by MAG and myelin.

Results

Blocking Inhibition by MAG/Myelin with db cAMP or Priming with Neurotrophins Is Transcription Dependent

Previously, we established that inhibition of axonal regeneration by MAG and myelin was blocked by elevating neuronal cAMP, either with the cAMP analog dibutyryl cAMP (db cAMP) or by exposing the neurons to various neurotrophins (NT) prior to when they are cultured on MAG or myelin (priming with NT) (Cai et al., 2001). Next we wanted to determine if the ability to block inhibition by MAG and myelin either with db cAMP or priming with NT is dependent on transcription.

Figure 1 shows that as reported before, neurite outgrowth from postnatal cerebellar neurons is inhibited by about 70% when grown on MAG-expressing CHO cells compared to control CHO cells not expressing MAG. Either addition of db cAMP directly to the cocultures or priming the neurons with the neurotrophin, brain-derived neurotrophic factor (BDNF) blocks this inhibition. Neither of these treatments has any effect on neurite outgrowth on control cells (Figures 1A and 1B). However, when an inhibitor of transcription, 5,6-dichloro-1-b-D-ribo-furanosyl-benzimidazole (DRB), is added either directly to the cultures when db cAMP is added or during priming with BDNF, the ability of either of these treatments to block inhibition is completely abrogated. That is to say, inhibition by MAG is the same as when no db cAMP or BDNF are added to the cultures. DRB has no effect on growth on control cells. Furthermore, Figure 2 shows that the ability of db cAMP and priming with BDNF to block inhibition of neurite outgrowth from cerebellar neurons on myelin is also transcription-dependent. On myelin, in the presence of db cAMP or after priming with BDNF, neurites are more than twice as long, but this improved growth is lost if DRB is included in the cultures. Therefore, the ability of elevated cAMP in neurons (either with db cAMP or by priming with BDNF) to block inhibition both by MAG and by myelin in general is transcription-dependent. It follows, then, that some gene(s) must be transcribed and protein(s) synthesized in response to elevated cAMP that are responsible for overcoming the inhibition by MAG/myelin.

The Enzyme Arginase I Is Upregulated and Polyamine Synthesis Increases in Neurons in Response to Either db cAMP or BDNF

Recently, Arg I was shown to be one of the rate-limiting enzymes in the synthesis of polyamines (Kepka-Lenhart et al., 2000) (Li et al., 2001). Furthermore, previous studies implicated polyamines in affecting axonal growth, both in culture (Abe et al., 1997; Chu et al., 1995), and in vivo (Dornay et al., 1986; Kauppila, 1992). Arg I is a cytosolic enzyme, which in liver and in macrophages it is upregulated in response to elevated cAMP (Morris et al., 1998; Nebes and Morris, 1988). To determine if Arg I is upregulated in neurons when cAMP is elevated, cerebellar neurons were treated for various times with either db cAMP or BDNF and (1) the RNA was extracted and subjected to RT-PCR using primers specific for Arg I, or (2) the proteins were extracted, subjected to PAGE, and transferred to membranes that were then stained with an Arg I antibody (Esch et al., 1998). Figure 3A shows that there is a basal level of expression of Arg I RNA in cerebellar neurons. When the neurons are treated with either db cAMP or BDNF before extraction of RNA, Arg I mRNA increases by at least 2-fold but never reaches the high levels found in liver (Figure 3A). Importantly, after treatment with either BDNF or db cAMP expression of Arg I, protein is increased by about 5-fold (Figure 3B). By 3 hr after treatment with either BDNF or db cAMP, the expression of Arg I protein, has reached maximum levels, which is sustained for at least 21 hr. By 24 hr, the level of expression has decreased slightly. The increase in Arg I expression is not due to a loading difference on the gel because the antibody used stains another protein of about 60 kDa nonspecifically, and the staining of this protein is unaffected by treatment with either db cAMP or BDNF (Figure 3B). Therefore, the increase in expression of Arg I protein in response to elevation of cAMP in neurons is specific.

To assess if polyamine synthesis is increased in neurons as a consequence of upregulation of Arg I by cAMP, levels of putrescine, spermidine, and spermine were measured after treatment with db cAMP or BDNF. Figure 4 shows that at both 6 and 18 hr after treatment with

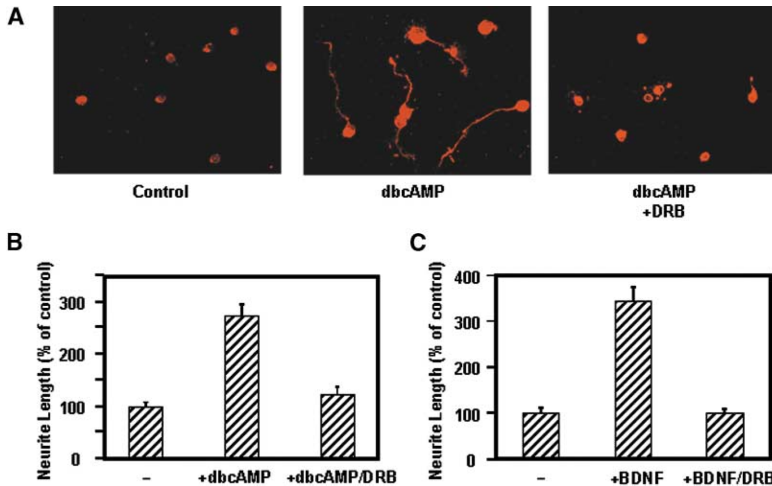

Figure 2. The Ability of db cAMP and BDNF to Overcome Inhibition by Myelin Is Transcription Dependent

Cerebellar neurons (P5) were isolated and either added directly to a substrate of myelin in the presence of db cAMP (1 mM) with and without DRB (5 μM) as indicated (A and B), or after being primed overnight with BDNF (200 ng/ml) (C), after which they were fixed and immunostained for GAP43. In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM) for at least four separate experiments.

db cAMP, the putrescine levels have increased by about 2-fold. For cerebellar neurons treated with BDNF, putrescine levels have doubled by 6 hr, and although still significantly elevated at 18 hr, are slightly decreased. Spermine and spermidine are also increased in response to elevated cAMP, but levels of these polyamines are higher at 18 hr than at 6 hr posttreatment (results not shown). This is consistent with spermidine and spermine being synthesized downstream of putrescine.

Overexpression of Arginase I Is Sufficient to Block Inhibition by MAG and Myelin

To determine if the upregulation of Arg I in response to cAMP is sufficient to overcome inhibition of regeneration by MAG/myelin, cerebellar neurons were infected with replication-incompetent adenoviral constructs containing the cDNA for Arg I. These viral constructs also contained the cDNA for green fluorescent protein (GFP). Cerebellar neurons were isolated, plated onto poly L-lysine and infected with viral constructs, and cultured overnight to allow expression of the transgene. Figure 5A is a Western showing expression of Arg I in cultures infected with either the Arg I cDNA-containing virus or control virus without Arg I cDNA. As can be seen when the Arg I cDNA-containing virus is used, there is an abundance of Arg I protein expression 24 hr after infection, while under these same conditions, expression of endogenous Arg I is undetectable in the control-infected neurons (Figure 5A). After culture for 24 hr, the infected cells were then transferred onto MAG-expressing CHO cells, control CHO cells, or myelin and cultured for 18–24 hr further before being fixed, stained, and neurite length measured. For these cerebellar neurons, the rate of infection is about 25%. Therefore, to ensure that only those neurons infected with virus were included in the analysis, only neurites from those neurons that were both GAP43 positive and GFP positive were measured

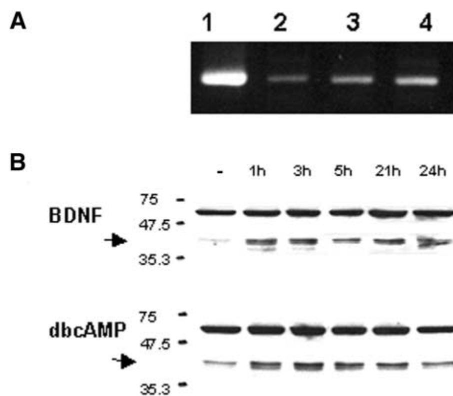

Figure 3. Arg I Is Upregulated in Cerebellar Neurons in Response to BDNF or db cAMP

Cerebellar neurons (P5) were plated onto poly L-lysine and cultured for various times, as indicated, with either BDNF (200 ng/ml) or db cAMP (1 mM).

(A) RNA was isolated, reverse transcribed, and subjected to RT PCR using Arg-I-specific primers before being separated in an agarose gel. Lane 1, RNA from liver; lane 2, RNA from untreated neurons; lane 3, neurons treated with BDNF for 18 hr; lane 4, neurons treated with db cAMP for 18 hr.

(B) Western blot of lysates from neurons treated with BDNF (top) or db cAMP (bottom) and immunostained with an Arg-I-specific antibody. Each lane was loaded with 50 μg of total protein. Times of incubation are indicated across the top. Molecular weight markers are indicated on the left. The arrow indicates Arg I protein.

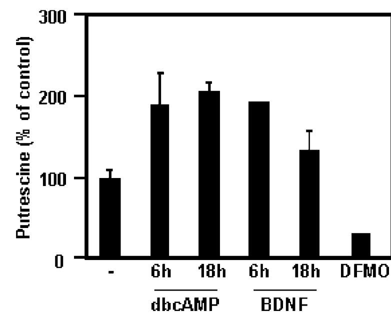

Figure 4. Synthesis of Putrescine Is Increased in Response to db cAMP or BDNF

Cerebellar neurons (P5) were plated onto poly L-lysine and treated with either db cAMP (1 mM), BDNF (200 ng/ml), or DFMO (1 mM) for various lengths of time as indicated and then lysed and putrescine content measured by HPLC. Results are expressed as a percentage of putrescine in untreated neurons ± SEM. Each measurement was carried out in triplicate, at least two times.

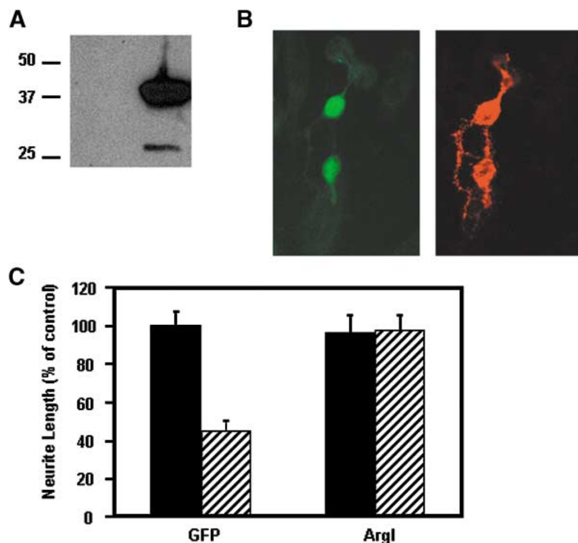

Figure 5. Overexpression of Arg I Is Sufficient to Overcome Inhibition by MAG and Myelin

Cerebellar neurons (P5) were isolated and infected with adenovirus vectors containing the cDNA for Arg I and/or the cDNA for GFP. 24 hr after infection, the neurons were either lysed and subjected to Western blotting or transferred to MAG-expressing CHO cells, control CHO cells, or myelin before being fixed and stained for GAP 43 and viewed for GFP.

(A) Western blot of neurons infected with Arg-I-cDNA-containing virus (right lane) or with control virus (left lane). Numbers refer to position of the molecular weight markers.

(B) Image of neurons positive for both GFP (left) and GAP43 (right).

(C) Measurement of neurite length from neurons infected with control virus (GFP) or with virus-containing Arg I cDNA (Arg I), grown on either MAG-expressing CHO cells (stripped bars) or control CHO cells (black bars) before being fixed and stained for GAP43. In each experiment, the mean length of the longest GAP43/GFP-positive neurite for 180–200 neurons was measured (± SEM). Each experiment was carried out at least three times.

(Figure 5B). It was found that infection of cerebellar neurons with the control virus expressing only GFP had no effect on the ability of MAG (Figure 5C) or myelin (data not shown) to inhibit neurite outgrowth. In contrast, neurons infected with the Arg-I-cDNA-containing virus were not inhibited by MAG or myelin. Neurite outgrowth in the Arg I-expressing neurons on MAG-expressing cells was the same as on the control CHO cells not expressing MAG. On myelin, neurite length was equivalent to neurites when the neurons were grown in the presence of db cAMP (20 ± 2 μm and 19 ± 2.3 μm, respectively). These results suggest that upregulation of Arg I alone, in the absence of elevation of cAMP, is sufficient to overcome inhibition of axonal growth by MAG and myelin.

Inhibitors of Polyamine Synthesis Block the Ability of Elevated cAMP to Overcome Inhibition by MAG and Myelin

Both Arg I and ornithine decarboxylase (ODC) are rate-limiting enzymes in the synthesis of polyamines from arginine. A specific and potent inhibitor of Arg I is N (omega)-hydroxynor-L-arginine 5 (NOHA) (Boucher et al., 1994) and of ornithine decarboxylate is DL-2-difluoromethyl-ornithine (DFMO) (Slotkin et al., 1982), each of which block the synthesis of polyamines. These two inhibitors were each added separately, either directly to the cocultures when db cAMP was used or during priming when BDNF was used. At a concentration of 1 mM, either NOHA or DFMO completely blocked the ability of db cAMP, or priming with BDNF, to overcome inhibition of neurite outgrowth by MAG and myelin (Figures 6A–6D). Neither inhibitor had an effect on growth on control cells, on MAG-expressing cells, or on myelin (Figure 6). This strongly suggests that synthesis of putrescine, and possibly other polyamines, plays a role in the ability of cAMP to overcome inhibition of MAG and myelin. To test if this is the case, putrescine was included in the cultures at the same time as NOHA or DFMO to determine if the improved growth induced by elevation of cAMP could be restored. As shown in Figure 6, putrescine was able to overcome the DFMO and NOHA block of the cAMP effect induced by either db cAMP or BDNF.

Priming with Polyamines Blocks Inhibition by MAG and Myelin

The question now raised is whether addition of polyamine alone is sufficient to block inhibition by MAG and myelin or whether an elevation of cAMP is also required to activate another signaling pathway, apart from putrescine synthesis. To test this possibility, putrescine was added to cultures when the neurons were grown on MAG-expressing cells, control cells, and on myelin. As can be seen in Figure 7, addition of putrescine alone can indeed block the inhibition by both MAG and myelin in a dose-dependent manner, with the maximum block of inhibition reached at a putrescine concentration of about 40 μM. Putrescine alone had no effect on neurite outgrowth from neurons grown on control cells or on poly L-lysine (results not shown). However, the block of MAG's inhibition was never complete as growth never reached that of neurons on the control CHO cells. The maximum reversal of inhibition was to about 60% of control. In contrast, if the neurons were primed with putrescine overnight before being plated on the MAG cells, inhibition was also blocked in a dose-dependent manner. But now, at 40 μM, inhibition by MAG was completely blocked; growth was the same as on control cells. Likewise, growth on myelin improved in a dose-dependent manner and, at a concentration of 40 μM, neurites were about twice as long if putrescine was added directly to the media without priming (Figure 7B). If instead the neurons were primed overnight with putrescine before being cultured on myelin, neurites were again longer, and the effect was dose-dependent. However, after priming at 40 μM putrescine, neurites were now more than four times longer than from neurons primed without putrescine (Figure 7B). The effect of priming with 40 μM putrescine on neurite outgrowth on myelin is even greater than that observed when db cAMP is added directly to the media or the neurons were primed with BDNF (Compare Figures 6B and 6D with Figure 7B). The putrescine effect is downstream of cAMP/PKA because inclusion of a PKA inhibitor in the assay has no effect on the ability of putrescine to overcome inhibition (Figure 7C). Similarly, there was no difference in neuronal survival when grown on myelin in the presence or absence of putrescine; under both conditions, about 80% of neurons survive (results not

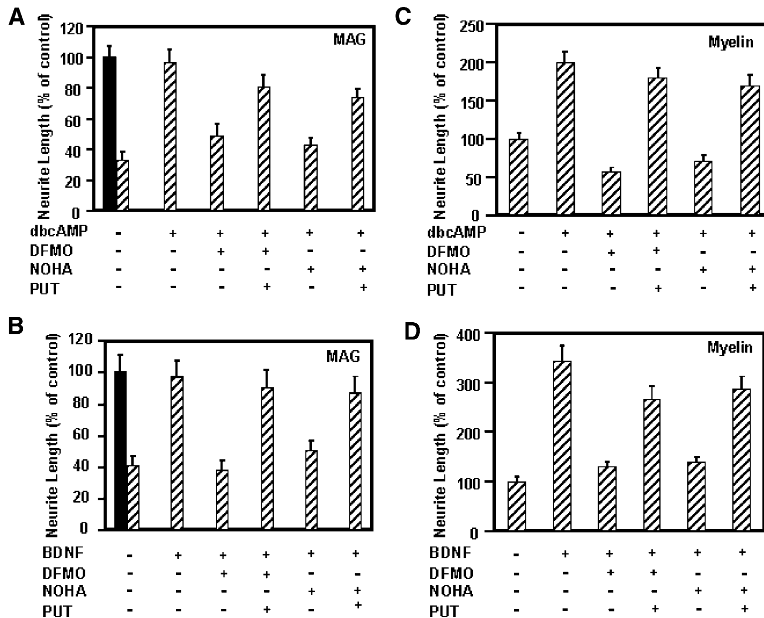

Figure 6. Blocking Polyamine Synthesis Overcomes the cAMP Effect on Neurite Outgrowth on MAG and Myelin, and Putrescine Restores It

Cerebellar neurons (P5) were isolated and cultured on (A and B) MAG-expressing CHO cells (stripped bars), control CHO cells (black bars), or (C and D) myelin, and cultured overnight before being fixed and stained for GAP43. Where indicated, db cAMP (dbcA) (1 mM), DFMO (1 mM), or putrescine (40 μM) were added directly to the cocultures or when being primed (24 hr) with BDNF (200 ng/ml). In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured ($\pm$ SEM) for at least three separate experiments.

shown). Taken together, these results demonstrate that putrescine alone can block the inhibition by MAG and myelin.

Arginase I in DRG Neurons Is Downregulated with Development that Coincides with the Switch from Promotion to Inhibition by MAG and Myelin

Previously, we showed that dorsal root ganglion (DRG) neurons switch their response to MAG and myelin with age (DeBellard et al., 1996). Up to about postnatal day (P)4, DRG neurons are promoted by MAG and will extend neurites on myelin. Sharply at about P4, this response changes, and DRG neurons older than P4 are inhibited by MAG and will not extend neurites on myelin. We also showed that this switch in response to MAG and myelin is dictated by a spontaneous decrease in the endogenous neuronal levels of cAMP (Cai et al. 2001). Next we showed that between P3 and P5, expression of Arg I protein in DRG neurons drops spontaneously and precipitously by about 5-fold (Figure 8A). The level of Arg I in DRG neurons remains at this low level through to adult. Consistent with the higher levels of Arg I in young DRG neurons, the ability of P1 DRG neurons to grow well on MAG is blocked by either the Arg I inhibitor, NOHA, or the ODC inhibitor DFMO. Addition of putrescine to these young neurons had no effect on growth on MAG cells or control cells (Figure 8B). Conversely, overexpression of Arg I in P7 DRG neurons in which endogenous levels are low overcomes inhibition by MAG (Figure 8C). These results suggest that a drop in expression of Arg I in DRG neurons is likely to be a consequence of the spontaneous decrease in cAMP and contributes to the switch in the response of these neurons from promotion to inhibition by MAG and myelin.

Discussion

The ability to alter CNS axons intrinsically such that they regenerate after injury in the adult would be a major advance in the development of therapies for spinal cord injury and general brain damage. We demonstrate here that overexpression of Arg I or elevation of polyamines are each sufficient to overcome the inhibition of regeneration by MAG and myelin, a strong indication that similar strategies can support regeneration in vivo. We also show that upregulation of Arg I and synthesis of polyamines are triggered by elevated cAMP, which we and others previously showed was sufficient not only to overcome inhibition by MAG and myelin, but also for dorsal root spinal axons to regenerate in vivo and for the regeneration of neonatal spinal axons (Cai et al., 2001; Qiu et al., 2002; Neumann et al., 2002). Consistent with these results, Arg I levels are high in young DRG neurons that are promoted by MAG and myelin. Moreover, if polyamine synthesis is blocked in young DRG neurons, so too is promotion. Conversely, in older DRG neurons that are inhibited by MAG and myelin, Arg I levels are lower. Overexpression of Arg I in these neurons neutralizes this inhibition. The decline in Arg I levels in DRG neurons with development parallels the decline in endogenous cAMP levels we recorded before. Together, these results provide insight into the molecular downstream consequences of elevated cAMP that results in regeneration. From the likely global effects of elevated cAMP, we can now move to a more focused approach to encourage regeneration in vivo.

Arginase I has not been extensively characterized in the nervous system. Liver and macrophages contain both the cytosolic isoform Arg I and the mitochondrial isoform Arg II. Although each isoform is differentially regulated, both are upregulated in response to cAMP (Morris et al., 1998; Nebes and Morris, 1988). What is particularly important is that Arg I has been shown to be a neuronal anti-apoptotic factor (Esch et al., 1998). Protein synthesis, required for apoptosis, is inhibited by depleting neuronal arginine levels. Thus, upregulation or overexpression of Arg I in neurons of the damaged nervous system would have two significant effects on neurons: preventing cell death and promoting regeneration through an extracellular environment that is inhibi-

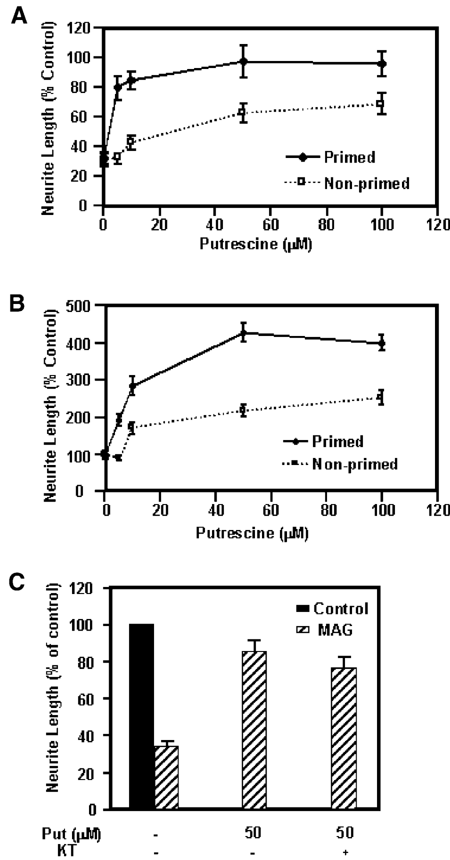

Figure 7. Putrescine Alone Can Overcome Inhibition by MAG and Myelin in a Dose-Dependent Manner

Cerebellar neurons (P5) were isolated and either primed overnight with various concentrations of putrescine (solid line), or putrescine was added directly to the cocultures (nonprimed, broken line) of neurons on MAG-expressing or control CHO cells (A) or on myelin (B). The PKA inhibitor, KT5720 (200 nM), was included when priming with putrescine (100 μM) before the neurons were grown on MAG-expressing (stripped bars) or control (black bars, 0 CHO cells. In each experiment, the mean length of the longest GAP43-positive neurite for 180–200 neurons was measured (± SEM) for at least three separate experiments. Results are represented as percent of control, which for (A) and (C) is control CHO cells and for (B), neurons grown on myelin in the absence of putrescine.

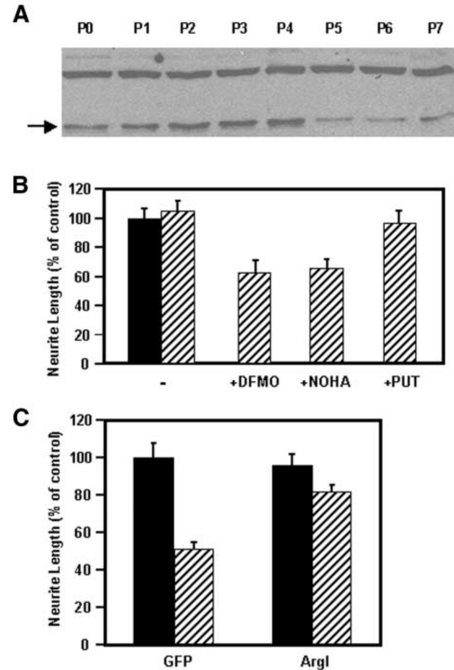

Figure 8. Arg I Levels Dictate the Switch from Promotion to Inhibition by MAG of DRG Neurons during Development

(A) DRG neurons from P0 to P7 animals were isolated, lysed, subjected to Western blotting, and immunostained for Arg I. Arrow indicates Arg I protein.

(B and C) P0 (B) or P8 (C) DRG neurons were isolated and cultured overnight on either MAG-expressing CHO cells (stripped bars) or control CHO cells (black bars) before being fixed and stained for GAP43. For the P0 DRGs (where indicated) DFMO (5 μM), NOHA (0.5 mM), or putrescine (100 μM) were included in the cultures. The P8 DRG neurons were infected with either control virus (GFP) or virus-containing Arg I cDNA (Arg I) prior to coculture on the CHO cells. In each experiment, the mean length of the longest GAP43-positive (B) or GAP43/GFP-positive (C) neurite for 180–200 neurons was measured (± SEM) for at least three separate experiments.

tory. Interestingly, cAMP by itself has also been shown to be sufficient to promote survival of certain types of neurons (Hanson et al., 1998), as well as augmenting the neurotrophin effects on others (Meyer-Franke et al., 1995, 1998). We can speculate that these effects are also mediated through an upregulation of Arg I.

Based on our latest findings, we are extending our hypothesis on the mechanism of action of cAMP in overcoming inhibition and promoting regeneration (Cai et al., 1999). We propose that a significant consequence of Arg I upregulation by cAMP is the synthesis of polyamines. The observations that support this are: first, that an inhibitor of either Arg I or ODC can block both the db cAMP and the BDNF effect in overcoming inhibition by MAG/myelin. Ornithine decarboxylase is directly responsible for synthesis of putrescine from ornithine. Although it was previously believed that ODC was the sole rate-limiting enzyme in the synthesis of polyamines from arginine, recent experiments have shown that Arg I is also rate-limiting (Kepka-Lenhart et al., 2000) (Li et al., 2001). In the experiments described here, Arg I does indeed appear to be rate-limiting because of its effect during development and because under these conditions, there is no change in ODC expression with either db cAMP or BDNF (results not shown). Second, putrescine both overcomes the block of Arg I and ODC inhibition and alone can overcome inhibition by MAG/myelin in a dose-dependent manner (Figures 6, 7, and 8). Intriguingly, putrescine's effect in reversing inhibition is enhanced with an overnight incubation before neurons are cultured on MAG or myelin. The effect could be due to the slow uptake of this highly charged molecule across the plasma membrane; when added to cultures at the same time as inhibitors, intracellular putrescine concentrations may be too low to achieve a maximal effect. Alternatively, it is possible that polyamines trigger another round of transcription and that synthesis of another unidentified protein(s) is required to overcome inhibition. The lag time for induction of new gene expression would require a longer period for maximum effect

of putrescine on inhibition. At this point in our investigations, because putrescine is readily converted to spermadine and then on to spermine, we do not know if it is indeed putrescine or the subsequently synthesized spermadine and spermine that is/are effecting the regeneration.

Although polyamine synthesis is sufficient to block inhibition, it is unlikely that it is the only pathway triggered by elevated cAMP levels. Many pathways known to be activated by cAMP (Montminy, 1997) could be stimulated in parallel, with each contributing some measure to overcoming inhibition. If this were the case, however, each pathway would only be minimally activated, resulting in a threshold effect and overcoming inhibition only when all pathways become active. Blocking any one pathway would diminish the total cAMP effect to below threshold, and inhibition would not be overcome. More reasonably, stimulating a single pathway maximally would be sufficient alone to block inhibition by MAG/myelin, which is what we see for overexpression of ArgI/polyamines.

Consistent with our observation that elevation of cAMP can overcome inhibition of MAG and myelin, others have shown that cAMP can change the repulsion of growth cones by MAG and myelin in general (Song et al., 1998). In these assays, however, the effects are acute, with a repulsive turning response to MAG and myelin occurring within an hour. In this turning assay, elevation of cAMP reverses the repulsion to attraction in the same time frame. Given the short time required to shift from repulsion to attraction, it is unlikely that this particular cAMP effect requires new transcription. Instead, activated PKA could be affecting the cytoskeletal dynamics directly, resulting in more immediate effects. Further work will be needed to determine whether the effect of cAMP on reversing growth cone turning and overcoming inhibition share mechanisms or are two independent events.

Polyamines have been implicated in nervous system development, axonogenesis, and regeneration (Chu et al., 1995; Dornay et al., 1986; Kauppila, 1992; Slotkin and Bartolome, 1986; Slotkin et al., 1982). The study presented here extends that work significantly by demonstrating that polyamines can overcome inhibition, rather than just accelerating regeneration, as previously suggested. The important question now is, how do polyamines bring about this effect? Although there are numerous reports of polyamine influence in the nervous system, none have pinpointed the mechanism behind their effects. In other tissue, polyamines have been shown to affect a variety of cell events (Cohen, 1998), including both DNA and protein synthesis. One possibility, then, is that polyamines may trigger a round of transcription to overcome inhibition by MAG/myelin. Polyamines also are known to have both short- and long-term effects on the behavior of the cytoskeleton. In studies on gastric mucosal healing, polyamines directly affect the polymerization and organization of microtubules within hours of treatment (Banan et al., 1998). This in turn promotes cell migration, a necessary step in this healing process. This is supported by another report showing that polyamines have a significant effect on the promotion of microtubule assembly from purified brain tubulin (Wolff, 1998). In these studies, the highly cationic polyamines are believed to promote assembly by neutralizing the anionic C termini of tubulin, so allowing the α and β monomers to come together more readily. It is possible, then, that polyamines directly affect the cytoskeleton to overcome inhibition. Furthermore, in the long-term (24–72 hr after treatment), polyamines increase the expression of genes encoding the major cytoskeleton proteins (Kaminska et al., 1992). It is conceivable that polyamines affect regeneration though both short and long term effects on the cytoskeleton. Finally, polyamines have been recently shown to affect both inward rectifying potassium channels and glutamate receptors in neurons (Chao et al., 1997; Kashiwagi et al., 1997; Williams, 1997; Williams et al., 1994). Given the role of glutamate and its receptors in synaptic plasticity (Abbott and Nelson, 2000; Gnegy, 2000), which also involves process outgrowth, it is reasonable to suspect that polyamines might overcome inhibition by MAG/myelin through a direct effect on these channels or receptors.

It is not known where polyamines exert their effect: inside or outside the neuron, at the cell body, or at the growth cone. We see an effect of polyamines after either increasing their concentration through an overexpression of Arg I inside the neuron as well as when they are added to culture media. Although polyamines are highly charged, they do cross the membrane in either direction. Moreover, polyamines have been shown to be transported down and to diffuse from the axon (Ingoglia et al., 1982; Lindquist et al., 1985). Clearly, the possibility exists that polyamine effects emerge through a variety of mechanisms, many of which may contribute to overcoming inhibition.

In summary, we have shown that elevation of cAMP leads to an upregulation of Arg I and the synthesis of polyamines, which overcome inhibition of axonal regeneration by MAG and myelin. In addition, the spontaneous growth of young neurons on MAG/myelin is also dependent on this Arg I/polyamine mechanism. These results reveal not only a novel mechanism to overcome inhibition, but also point to new and specific therapeutic strategies to change the intrinsic ability of neurons to grow through an inhibitory CNS environment and thereby encourage regeneration in vivo.

Experimental Procedures

Neurite Outgrowth on Cells or Myelin

For myelin membranes (Norton and Poduslo, 1973), wells of an eight-chamber tissue culture slide (Lab-Tek) were coated with 16.6 μg/ml poly L-lysine at room temperature for 1 hr. Rat CNS myelin at 0.5–1.0 μg total protein/well was dried overnight onto the coated wells and used as a substrate (Mukhopadhyay et al., 1994; Shen et al., 1998). Monolayers of control and MAG-expressing CHO cells (Mukhopadhyay et al., 1994) were grown to confluency in individual chambers of an eight-chamber tissue culture slide (Lab-Tek). Cerebellar and DRG neurons were isolated as described previously (DeBellard et al., 1996). Where indicated neurons were plated on poly L-lysine and primed overnight with BDNF (200 ng/ml) or putrescine (10–100 mM) before being transferred to myelin or the CHO cells. The neurite outgrowth assay was carried out as described previously (Mukhopadhyay et al., 1994) with the following modifications. 5×10^4 neurons were plated onto immobilized myelin and 2×10^4 neurons were used for the CHO cell monolayers. Where indicated, db cAMP (1 mM), DRB (5 μM), DFMO (1–5 mM), NOHA (0.2–0.5 μM), putrescine (10–100 μM), or BDNF (200 ng/ml) were added directly to cultures or during priming. After 16–18 hr of incubation, the cul-

tures were fixed for 30 min with 4% paraformaldehyde, permeabilized with ice-cold methanol, and immunostained with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis and G. Wilkins, Imperial College, London) as previously described (Mukhopadhyay et al., 1994). The slides were mounted in Permafluor (Immunon) and viewed under a fluorescence microscope. The length of the longest neurite for each GAP43-positive neuron for the first 180–200 neurons encountered when scanning the slide in a systematic manner was determined using an Oncor image analysis program.

Detection of Arg I Gene Expression

Total RNA was isolated from 2×10^6 cerebellar neurons, which were either treated with BDNF (200 ng/ml) or db cAMP (1 mM) overnight, using an RNeasy kit (Qiagen) following the manufacturers' instructions. cDNA was synthesized from total RNA using random hexamers and reverse transcriptional enzyme (Stratagene) at 37°C for 2 hr. After which, Arg I cDNA was amplified using the following specific primers (Gibco BRL) that spanned nucleotide 197 to 700 of Arg I cDNA (Accession number NM017134): 5′ GTC CCC AAT GAC AGC CCC 3′ and 5′ CTT TTC TTC CTT CCC AGC AG 3′ using the polymerase chain reaction (PCR) at 35 cycles These primers were synthesized by GibcoBRL. Arg I cDNA was detected in a 1% agarose gel, stained with ethidium bromide.

Immunodetection of Arg I

Cerebellar or DRG neurons of different ages were lysed in 50 mM Tris-HCl (pH 7.4) containing 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM vanadate, and the following antiproteases: 1 μg/ml each of leupeptin, aprotinin, and pepstatin. The cell lysates were kept on ice for 15 min and centrifuged at $14{,}000 \times g$ for 10 min. Protein concentration was measured in the supernatant with a Bio-Rad kit (Bio-Rad Labs, Hercules, CA). Normalized lysates were boiled for 5 min, after which they were subjected to SDS-PAGE in a 12% polyacrylamide gel, transferred to nitrocellulose membranes, and immunostained with a rabbit polyclonal antibody against Arg I (Esch et al., 1998) at 1:5000. Detection was with an enhanced chemiluminescence (ECL) detection system, according to the manufacturers' instructions (Amersham).

Measurement of Polyamine

5×10^6 postnatal cerebellar neurons were plated onto poly L-lysine coated six-well plates. BDNF (200 ng/ml), dbcAMP (1 mM), or DFMO (1 mM) were added and incubated for various times as indicated. Cells were washed and lysed in 100 μl of 1.5 M$HClO_4$. Three lysates were pooled and the reaction was stopped by adding 150 μl of neutralizing buffer (2 M potassium bicarbonate). After centrifugation, polyamines in the supernatant were measured by HPLC carried out by a service (Dr. G. Wu, Department of Animal Science, Texas A&M University).

Cell Survival Assay

The number of live and dead cells was estimated using a Live/Dead Viability/Cytotoxicity kit, according to the manufacturer's instructions (Molecular Probes). Briefly, P5 cerebellar or P6–P8 DRG neurons were plated at 50,000 cells per myelin-coated well of an eight-chamber slide. The neurons were cultured overnight in the presence or absence of BDNF (200 ng/ml) or putrescine (10 μM or 50 μM), after which they were incubated with the vital dye Calcein AM and ethidium homodimer-1. At least 200 neurons were scored for each condition, and each experiment was carried out at least twice.

Adenoviral Infection of Neurons

Postnatal cerebellar neurons or DRG neurons of various ages as indicated and were plated on poly L-lysine-coated wells. Neurons were infected with recombinant adenovirus containing GFP cDNA (He et al., 1998) and Arg I cDNA or only GFP cDNA as control at a final concentration of 10^8 PFU/ml. 1 hr after infection, media was changed and neurons were maintained for 1 day to recover and to allow gene expression. Infected neurons were plated onto MAG-expressing cells, control CHO cells, or myelin substrate, and the neurite outgrowth assay was carried out as described above. After overnight incubation, cultures were fixed and immunostained for GAP43. The longest neurite from 180–200 GFP/GAP43 double-positive neurons was measured.

Acknowledgments

We thank Dr. Roger Persell for critically reading this manuscript and Dr. Lloyd Williams for his assistance with the image analysis. This work was supported by grants from the National Multiple Sclerosis Society, NIH (NINDS NS 37060 to M.T.F. and NS40591 to R.R.R.), a grant to support a Specialized Neuroscience Research Program (SNRP) from NIH (NINDS and NCRR; NS41073) at Hunter College, and a core facility grant from Research Center for Minority Institutions.

Received: December 11, 2001
Revised: June 6, 2002

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