Role of Muscle Insulin-like Growth Factors in Nerve Sprouting: Suppression of Terminal Sprouting in Paralyzed Muscle by IGF-binding Protein 4

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Abstract. The protracted absence of muscle activation initiates complex cellular and molecular reactions aimed at restoring functional neuromuscular transmission and preventing degenerative processes. A central aspect of these reactions is the sprouting of intramuscular nerves in the vicinity of inactivated muscle fibers. Sprouts emerging from terminal nerve branches and nodes of Ranvier can reestablish functional contacts with inactive muscle fibers, and this is an essential restorative process in pathological conditions of the neuromuscular system. Due to their rapid upregulation in inactive skeletal muscle fibers and their ability to induce nerve sprouting in adult muscle, insulin-like growth factors (IGFs) are candidate signaling molecules to promote restorative reactions in the neuromuscular system. In this study we have exploited the high affinity and specificity of IGF-binding protein 4 (IGF-BP4) and IGF-BP5 for IGF1 and IGF2 to determine whether these growth factors are involved in the nerve sprouting reaction in paralyzed skeletal muscle.

In tissue culture experiments with sensory- and motoneurons we demonstrate that the neurite promoting activity of IGF1 is blocked by IGF-BP4, and that a similar IGF-BP-sensitive activity is detected in muscle extracts from paralyzed, but not from control muscle. In in vivo experiments, we show that local delivery of IGF-BP4 to Botulinum toxin A-paralyzed skeletal muscle effectively prevents nerve sprouting in that muscle. Our findings indicate that muscle IGFs play an essential role in intramuscular nerve sprouting. In addition, these findings suggest that IGFs are major signaling factors from inactivated muscle to promote local restorative reactions, including interstitial cell proliferation and nerve sprouting.

The neuromuscular system reacts to the prolonged absence of muscle activation with a complex set of cellular reactions aimed at preventing degenerative processes and at restoring normal activity. Knowledge about the molecular and cellular mechanisms involved in these restorative reactions is important to our understanding of the processes involved in neuromuscular junction development and maintenance. In addition, it is central to progress in the understanding and treatment of pathological situations involving the neuromuscular system; these include peripheral neuropathies, peripheral regeneration upon lesion, and motoneuron disease.

The reactions of the neuromuscular system to functional inactivation have been studied extensively and, as a consequence, much is known about the sequence of events involved, at the physiological, cellular, and molecular level. At the macroscopic level, inactivation leads to proliferation of muscle interstitial cells (Murray and Robbins, 1982; Connor and McMahan, 1987; Gatchalian et al., 1989), and to intramuscular sprouting of the remaining intact nerves (Holland and Brown, 1981; Brown, 1984). At the molecular level, gene expression is affected in most cells in the vicinity of inactive skeletal muscle fibers, including fibroblasts, terminal Schwann cells, and the muscle fibers themselves (Laufer and Changeux, 1989; Gatchalian et al., 1989; Tsay and Schmid, 1990; Eftimie et al., 1991). These local reactions are probably brought about by alterations in the set of proteins exposed on the surface of the reacting cells, and by diffusible factors.

The evidence for a role of muscle-derived diffusible factors in the reactions of the neuromuscular system to functional inactivation is mostly circumstantial. Thus a stimulus for intramuscular nerve sprouting probably spreads for ~100 μm from inactivated neuromuscular junctions (Brown et al., 1980; Slack and Pockett, 1981; Kuffler, 1989). Local proliferation of muscle interstitial cells, mainly in the vicinity of inactivated neuromuscular junctions, is likely to be triggered by muscle-derived diffusible activities, since an extensive basal lamina probably prevents direct surface interactions between inactivated muscle fibers and nearby interstitial cells. Finally, substantially elevated levels of neurite

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outgrowth producing activities were recovered in soluble protein extracts from paralyzed skeletal muscle (Henderson et al., 1983).

While the cellular source of these activities need not exclusively be the skeletal muscle fiber, it is clear that the signal(s) that initiate the restorative reactions must come from the inactivated muscle fibers.

Candidate muscle-derived signaling factors whose expression is upregulated by muscle inactivation include the insulin-like growth factors IGF1 and IGF2 (Ishii, 1989; Rechler and Nissley, 1990; Caroni and Schneider, 1994). There is substantial evidence suggesting that IGFs may be muscle-derived factors affecting the responses of the neuromuscular system to functional inactivation. Thus their expression is rapidly upregulated in Botulinum toxin paralyzed or denervated skeletal muscle (Ishii, 1989; Caroni and Schneider, 1994). Elevated levels of IGF1 mRNA were detected as early as 15 h after paralysis and peak levels were reached after about 36 h (Caroni and Schneider, 1994). Similar findings were obtained for the corresponding protein. IGF1 induction in skeletal muscle therefore parallels that of the myogenic transcriptional regulators MyoD1 and myogenin and of the α-subunit of the nicotinic acetylcholine receptor (Tsay and Schmid, 1990; Eftimie et al., 1991; Caroni and Schneider, 1994). It precedes or coincides with the interstitial cell proliferation reaction, one of the earliest macroscopically detectable cellular reactions in paralyzed muscle (Murray and Robbins, 1982; Connor and McMahan, 1987; Gatchalian et al., 1989). In skeletal muscle, IGF1 receptors are found on muscle fibers, fibroblasts, and motoneurons, indicating that these cells could be affected by the elevated levels of IGFs that are induced upon muscle inactivation (Shimizu et al., 1986; Tollefsen et al., 1989; Lewis et al., 1993). Furthermore, we have recently demonstrated that a signaling pathway involving IGF1 does operate from skeletal muscle to spinal motoneuron cell body (Caroni and Becker, 1992). This signaling pathway can affect the downregulation of motoneuron growth-associated proteins in neonatal rats at the time of synapse elimination. Finally, elevating the levels of intramuscular IGFs in adult skeletal muscle by local subcutaneous injections is sufficient to stimulate local interstitial cell proliferation and nerve sprouting (Caroni and Grandes, 1990; Lewis et al., 1993).

The biology of the IGFs is affected by highly specific binding proteins that bind to and modulate the activity of the growth factors in the extracellular space (Froesch et al., 1985; Baxter and Martin, 1989; Clemmons, 1990; Rechler and Nissley, 1990; Rosenfeld et al., 1990; Shimasaki and Ling, 1991). The IGF-binding proteins (IGF-BPs) used in this study, i.e., IGF-BP4 and IGF-BP5 bind to IGF1 and IGF2 with undistinguishable affinity (Kiefer et al., 1989). Corresponding K_A values determined in vitro with purified recombinant components were 10–100-fold higher than that of the IGF1 receptor for IGF1 and IGF2 (Kiefer et al., 1992). In biological assays with cultured cells, the inclusion of a 10-fold molar excess of IGF-BP over IGF is sufficient to block mitogenic and differentiation activities of the IGFs (Kiefer et al., 1992). In addition, IGF-BP4 and IGF-BP5 do not appear to bind with biologically relevant affinities to known growth factors other than IGF1 or IGF2 (Baxter and Martin, 1989; Clemmons, 1990; Rosenfeld et al., 1990; Shimasaki and Ling, 1991). Therefore, these IGF-BPs are highly potent and specific naturally occurring ligands of IGFs. As such, they may potentially be applied to specifically interfere with the actions of IGFs in vitro, and possibly also in vivo.

In the present study we have taken advantage of the strong and specific interaction between the IGFs and IGF-BP4 (or IGF-BP5) to determine whether muscle IGFs are involved in nerve sprouting in inactivated skeletal muscle. We report that the neurite outgrowth promoting activity in soluble protein extracts from paralyzed skeletal muscle is neutralized when IGF-BPs are included in the culture medium. In addition, local release of IGF-BP4 from an osmotic minipump implanted subcutaneously over paralyzed mouse gluteus muscle prevented intramuscular nerve sprouting. Together with our previous finding that locally applied IGF-BP5 prevents interstitial cell proliferation in paralyzed skeletal muscle (Caroni and Schneider, 1994), our results indicate that muscle IGFs are a central component of signaling in inactivated muscle. In addition, our findings suggest that muscle-derived IGFs play an essential role in the nerve sprouting reaction in paralyzed skeletal muscle.

Materials and Methods

Reagents

Human recombinant IGF-BP4 and IGF-BP5 were expressed in yeast and purified as described previously (Kiefer et al., 1992). For stock solutions, the IGF-BPs were dissolved at 100–300 μg/ml in PBS with 0.1% BSA; these solutions were then filtered through a sterile 0.22-μm filter and stored for periods of up to 2 months at 4°C. Purified Botulinum toxin A was a kind gift of V. Witzemann, Max Planck Institute, Heidelberg, FRG. Human recombinant IGF1 was a kind gift of J. Fischer, University of Zürich, Switzerland. The IGF1 derivative long^{3}R-IGF1 was from GroPep, Adelaide, Australia. In contrast to IGF1, it binds poorly to IGF-BPs, and displays elevated potency in in vitro and in vivo experiments. Mouse βNGF and bFGF were from Boehringer-Mannheim. Rat recombinant ciliary neurotrophic factor (CNTF)[1] was from Preprotek Inc., Rocky Hill, NJ. Purified brain-derived neurotrophic factor (BDNF) was a kind gift from Y.-A. Barde, Max Planck Institute for Psychiatry, Munich, FRG. Monoclonal antibody to 160-kD neurofilament protein was from Sigma Chem. Co. (St. Louis, MO). Biotin-conjugated goat-anti-mouse, and lucifer yellow-conjugated streptavidin were from Molecular Probes (Eugene, OR). Laminin was purchased from Collaborative Research (Bedford, MA). ALZET osmotic minipumps (model 1007D) were from ALZA Corp. (Palo Alto, CA).

In Vivo Experiments

20–25 *g* Balb/C mice were paralyzed locally with a single injection of 25 pg of Botulinum toxin A (Thesleff, 1989) into one gluteus muscle. Local paralysis developed within 24 h and lasted for at least 10 d. The extent of paralysis varied somewhat between animals: while most animals did retain some residual motility of the paralyzed leg, no toxin-treated animal was free of obvious signs of local paralysis when the toxin was applied at this dosage. When indicated, osmotic minipumps (1.5-cm length; inner volume of 100 μl; rate of delivery: 11 μl per day) were implanted subcutaneously over the gluteus muscle just before application of the Botulinum toxin.

Attention was paid to the positioning of the minipump: this was implanted anterior relative to the gluteus muscle, parallel to the body axis, ~0.5–1 cm laterally from the vertebrae column, and with its opening slightly anterior of the femur. Handling of the pumps was according to the recommendations of the manufacturer. For most experiments, IGF-BPs were diluted to a final concentration of 100 μg/ml in PBS with 0.1% BSA. In most cases, the position of the pump did not change in an obvious manner during the course of the experiment. 7 d after initiation of the experiments

1. *Abbreviations used in this paper*: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; E, embryonic day; DRG, dorsal root ganglia; IGF, insulin-like growth factor; IGF-BP, IGF-binding protein.

animals were sacrificed, the pumps carefully removed and emptying of the pump during the experiment verified by visual inspection, as recommended by the manufacturer. None of the osmotic minipumps appeared to have misfunctioned.

Because IGF-BPs are very stable at 37°C, no attempts were made to verify that the binding proteins in the minipumps were still active at the end of the experiment. For the combined silver esterase reaction, dissected gluteus muscles were maintained for 5 min in PBS with 5 mM EDTA, mounted for cryosectioning, and processed as described (Pestronk and Drachman, 1978). A section thickness of 50 μm was found to yield reproducible results with respect to reliable staining of terminal branches and sprouts. At least ten sections per muscle were processed and analyzed. Three evenly stained sections per muscle were further analyzed at a magnification of 500×. To determine the fraction of endplates with sprouts, all endplates from randomly selected fields were analyzed. Endplates with at least one sprout that clearly emerged from the endplate region and was longer than 5 μm were scored as positive. At least 100 endplates per muscle were analyzed, and values in Fig. 5 are the mean and standard deviation from five independent animals. Lengths of terminal sprouts were estimated with Image 1.4 (NIH) software. Briefly, an axiovert 10 microscope (Carl Zeiss, AG) was connected to a Macintosh IIcx computer through a S/W-CCD camera (model BC-2, AVT-Horn). Sprouts were treated as two-dimensional structures, and a total of at least 50 terminal sprouts from randomly selected fields were analyzed per muscle. Values in Fig. 5 are means and standard deviations from five independent animals. Where indicated, endplate areas were estimated by calculating ellipse areas from largest and smallest diameters, as deduced from data like those shown in Fig. 4. In this case, values are means and standard deviations from three independent animals (total of 75 endplates).

Soluble protein fractions from mouse gluteus muscles were prepared 4 d after initiation of the various treatments, as described (Henderson et al., 1983). Briefly, muscles were homogenized with a polytron in ice-cold calcium- and magnesium-free Hanks with 5 mM EDTA; 100,000 *g* (1 h) supernatants were collected. Protein concentrations were adjusted to 1 mg/ml and extracts were stored at −20°C. The 4-d interval between initiation of the treatment and collection of the muscles was selected based on the assumption that extracts may then contain highest levels of neurite-promoting activity (Henderson et al., 1983).

Cell Culture Experiments

Dissociated embryonic day (E) 8 chick dorsal root ganglia (DRG) neurons were isolated by a standard protocol. Briefly, ganglia were exposed to trypsin, and dissociated; cells were washed and plated at low density on laminin- and polyornithine-coated glass coverslips in DMEM with 10% FCS and 2 ng/ml of NGF. Where indicated, muscle extracts (1 μg/ml, final concentration), IGF-BPs, and long^{3}R-IGF1 were diluted into the culture medium at the time of plating. E6 chick and E15 rat spinal motoneurons were purified and cultured as described (Henderson et al., 1993; Camu et al., 1993). Chick motoneurons were cultured in enriched L15 in the presence of 2% horse serum, 2 ng/ml CNTF, and 10 ng/ml bFGF (Arakawa et al., 1990; Camu et al., 1993). For rat motoneurons, the culture medium was supplemented with 2 ng/ml of BDNF (Henderson et al., 1993). Motoneurons were plated at a density of ∼3,000 cells per 35-mm culture dish. For most experiments with DRG neurons, cultures were fixed in 4% paraformaldehyde 3 h after plating, and processed for immunocytochemistry as described (Widmer and Caroni, 1993). GAP-43 immunoreactivity was detected by incubation with the specific monoclonal antibody 5F10, followed by goat-anti-mouse biotin, and lucifer yellow-streptavidin, as described (Widmer and Caroni, 1993).

Neurite lengths were measured from photographs of stained (GAP-43, DRG neurons; neurofilament-160, chick motoneurons) or living (rat and chick motoneurons) cultures, using NIH Image 1.4 software. All neurons from randomly selected fields (250× [DRG] or 60× [motoneurons] magnification) with at least one neurite longer than a cell diameter were analyzed. Neurite length was defined as a neuron's longest neurite. In 3 h DRG cultures, most neurons had grown several neurites of comparable length. 50 neurons were analyzed per experiment and values in Fig. 1 and Table I are averages and standard deviations from the pooled data of 4 (DRG) or 3 (motoneurons) independent experiments. For 2 d, motoneuron survival values, all cells with neurites in 35-mm culture dishes were counted (two determinations, average value given in Table I). Growth cone areas and number of branch points per 100 μm were estimated from data like those shown in Fig. 2, *a–c*. All neurons from randomly selected fields whose neurites and growth cones could be assigned unambiguously were analyzed. For branch point per 100 μm values, all primary neurites (longest distance from cell body to neurite tip) were analyzed. Values are means and standard deviations from 100 neurons (two independent experiments).

Table I. Suppression by IGF-BP4 of an Activity in Protein Extracts from Paralyzed Muscle That Stimulates Neurite Outgrowth from Cultured Chick and Rat Spinal Motoneurons

Muscle extract	Neuron numbers		Neurite length (μm)	
	no BP	+IGF-BP4	no BP	+IGF-BP4
Chick motoneurons				
none	1081	1185	415 ± 145	470 ± 155
control muscle	1202	1151	580 ± 167	560 ± 151
paralyzed muscle	1010	1186	1012 ± 305	606 ± 186
Rat motoneurons:				
none	1452	1514	365 ± 111	337 ± 108
control muscle	1397	1470	399 ± 138	371 ± 131
paralyzed muscle	1597	1545	853 ± 276	390 ± 115

Approximately 3,000 purified spinal motoneurons were plated onto laminin-coated 35-mm culture dishes and 2-d cultures were analyzed. Neuron number values are averages from two independent experiments. Neurite length values are averages and standard deviations (total of 100 neurons from two independent experiments). 100 ng/ml of IGF-BP4 specifically suppressed the neurite-promoting activity in 1 μg/ml of protein extract from paralyzed muscle ($p < 0.0005$; Student's *t* test). Note that the binding protein did not detectably affect 2 d motoneuron survival in the presence of CNTF and bFGF (chick motoneurons), or BDNF (rat motoneurons). Also note that the binding protein did not significantly affect basal neurite outgrowth in the absence or in the presence of muscle extract from untreated control muscle.

Results

A Paralysis-induced Neurite Outgrowth Promoting Activity in Soluble Protein Fractions from Muscle Is Blocked by IGF-BP4

In a first series of experiments, we determined whether IGF-BPs may counteract the effects of IGFs on cultured neurons, and whether extracts from paralyzed muscle may contain IGF-BP-sensitive activities. IGFs are known to effectively promote neurite outgrowth in vitro when tested at concentrations as low as 100 pM. Neurons responsive to IGFs in neurite outgrowth assays include sympathetic, sensory, and motoneurons (Recio-Pinto et al., 1986; Caroni and Grandes, 1990). As shown in Fig. 1 *A*, 500 pM of purified human recombinant IGF1 effectively accelerated the initial rate of neurite outgrowth from dissociated E8 chick DRG neurons cultured on a laminin-coated substratum in the presence of 2 ng/ml of NGF. These concentrations of NGF are saturating with respect to the survival of DRG neurons, and the laminin presumably provided optimal substratum conditions for these neurons. The figure also shows that a 10-fold molar excess of purified recombinant human IGF-BP4 over IGF1 completely abolished the effect of the IGF on neurite outgrowth. On the other hand, no effects of IGF-BP4 on neuron numbers could be detected, in agreement with its inability to bind to NGF.

One to two months old mice were treated locally with a single 25 pg dose of purified Botulinum toxin A. The toxin was applied into one gluteus muscle, thus producing complete and lasting local paralysis. Four days after toxin treatment, animals were sacrificed and protein extracts from the paralyzed and from the control contralateral gluteus muscle were prepared. These were tested in the neurite outgrowth assay at protein concentrations of 1 μg/ml. As shown in Fig. 1(*A3*), the extract from the paralyzed muscle was approxi-

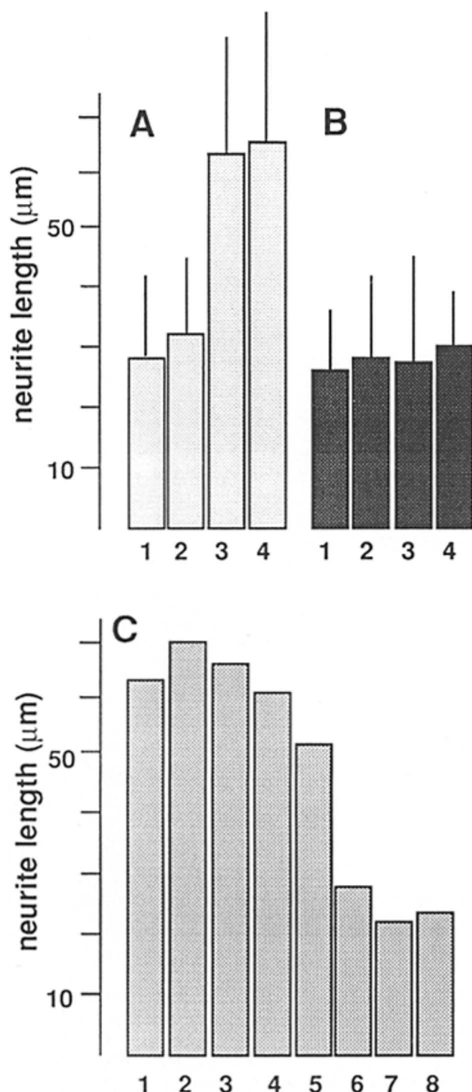

Figure 1. Suppression of the neurite outgrowth-promoting activity of IGF1 by IGF-BP4, and demonstration of IGF-BP4-sensitive neurite promoting activity in protein extracts from paralyzed muscle. Dissociated E8 chick DRG neurons were plated on a laminin-coated substratum in the presence of 2 ng/ml of NGF. Cells were fixed 3 h after plating and the average length of a neuron's longest neurite was determined. (*A* and *B*) Neurons cultured without (*A*) and with (*B*) 100 ng/ml of IGF-BP4. Further additions to the culture medium were: (*1*) no additions; (*2*) 1 μg/ml of soluble proteins from control adult mouse gluteus muscle; (*3*) 1 μg/ml of protein extract from gluteus muscle 4 d after paralysis with Botulinum toxin A; (*4*) 0.5 nM IGF1. Average values and corresponding standard deviations are given in the figure (total of 200 neurons analyzed per value; from four independent experiments with 50 neurons per experiment). A3 and A4 are significantly different from A1 and A2 and B1-4 ($p < 0.0005$; Student's *t* test). (*C*) Dose-dependent inhibition of the neurite-promoting activity in a protein extract from paralyzed muscle by IGF-BP4 (experimental conditions as in *A3*). The final concentrations of added IGF-BP4 were (*1*) none; (*2*) 2 ng/ml; (*3*) 5 ng/ml; (*4*) 10 ng/ml; (*5*) 25 ng/ml; (*6*) 100 ng/ml; (*7*) 200 ng/ml; 8,500 ng/ml. Values are the average of two independent experiments (50 neurons per experiment). 1 nM IGF-BP4 corresponds to ~25 ng/ml.

mately as effective as 500 pM IGF1 in stimulating neurite outgrowth in the DRG neuron assay. In contrast, no stimulation over the rate induced by 2 ng/ml of NGF was detected in the presence of 1 μg/ml of extracts from contralateral, non-paralyzed muscle, or from the gluteus muscle of an untreated animal. This finding is in agreement with previous reports that demonstrated the presence of elevated contents of neurite outgrowth promoting activity in soluble protein extracts from denervated muscle (Henderson et al., 1983). While earlier data were produced with mixed spinal cord-derived neuronal cultures, our findings now demonstrate that activities acting on chick DRG neurons are also present in the extracts from inactivated mouse muscle.

The data of Fig. 1 demonstrate that the neurite-promoting activity in the extract from paralyzed muscle could be neutralized by IGF-BP4 in a dose-dependent manner. 100 ng/ml of IGF-BP4 were sufficient to neutralize the activity present in 1 μg/ml of protein extract. The blocking activity was not restricted to IGF-BP4, since 100 ng/ml of human recombinant IGF-BP5 were equally effective in neutralizing the activity present in the muscle extract (data not shown). From these experiments we conclude that under our experimental conditions the neurite-promoting activity detected in soluble protein extracts from paralyzed mouse skeletal muscle depends on, and is probably due to IGFs.

Protein extracts from paralyzed muscle not only accelerated the initial outgrowth of neurites from DRG neurons, but also produced striking effects on neurite and growth cone morphology. As shown in Fig. 2, DRG neurons grown for 24 h in the presence of extract from paralyzed muscle had larger growth cones (average area: 164 ± 38 μm^2) and more branched neurites (5.4 ± 1.2 branchpoints per 100 μm) than their counterparts in the presence of extract from non-paralyzed muscle (growth cone area: 32 ± 15 μm^2; branchpoints per 100 μm: 1.9 ± 0.5). Significantly, this effect of the extract from paralyzed muscle was abolished when 100 ng/ml of IGF-BP4 were included in the culture medium (Fig. 2 *c*; with IGF-BP4: growth cone area: 45 ± 16 μm^2; branchpoints per 100 μm: 2.1 ± 0.6). On the other hand, IGF-BP4 had no apparent effects when neurons were grown in the presence of extract from control muscle (Fig. 2 *b*; with IGF-BP4: 29 ± 14 μm^2 and 2.2 ± 0.7 branchpoints per 100 μm) or of NGF alone (Fig. 2 *a*; 29 ± 17 μm^2 [no IGF-BP4] and 27 ± 15 μm^2 [with IGF-BP4]; 1.8 ± 0.7 [no IGF-BP4] and 2.0 ± 0.5 [with IGF-BP4] branchpoints per 100 μm). Therefore, muscle extracts from paralyzed mouse skeletal muscle contain activities promoting the growth, spreading, and branching of neurites from chick DRG neurons, and these activities are suppressed by IGF-BPs.

The IGF-BP-sensitive effect of protein extracts from paralyzed muscle on neurite outgrowth could also be detected with cultured chick and rat spinal motoneurons. As shown in Fig. 3 and Table I, 100 ng/ml of IGF-BP4 effectively suppressed the neurite-promoting activity in 1 μg/ml of protein extract from paralyzed muscle. Importantly, IGF-BP4 had no effect on neurite outgrowth in the absence of muscle extract. In addition, IGF-BP4 did not detectably affect motoneuron survival under any of the experimental conditions tested, including where no muscle extract was added to the culture medium. Under such experimental conditions more than 60% of the motoneurons die in the absence of appropriate survival-promoting factors (Arakawa et al., 1990; Hen-

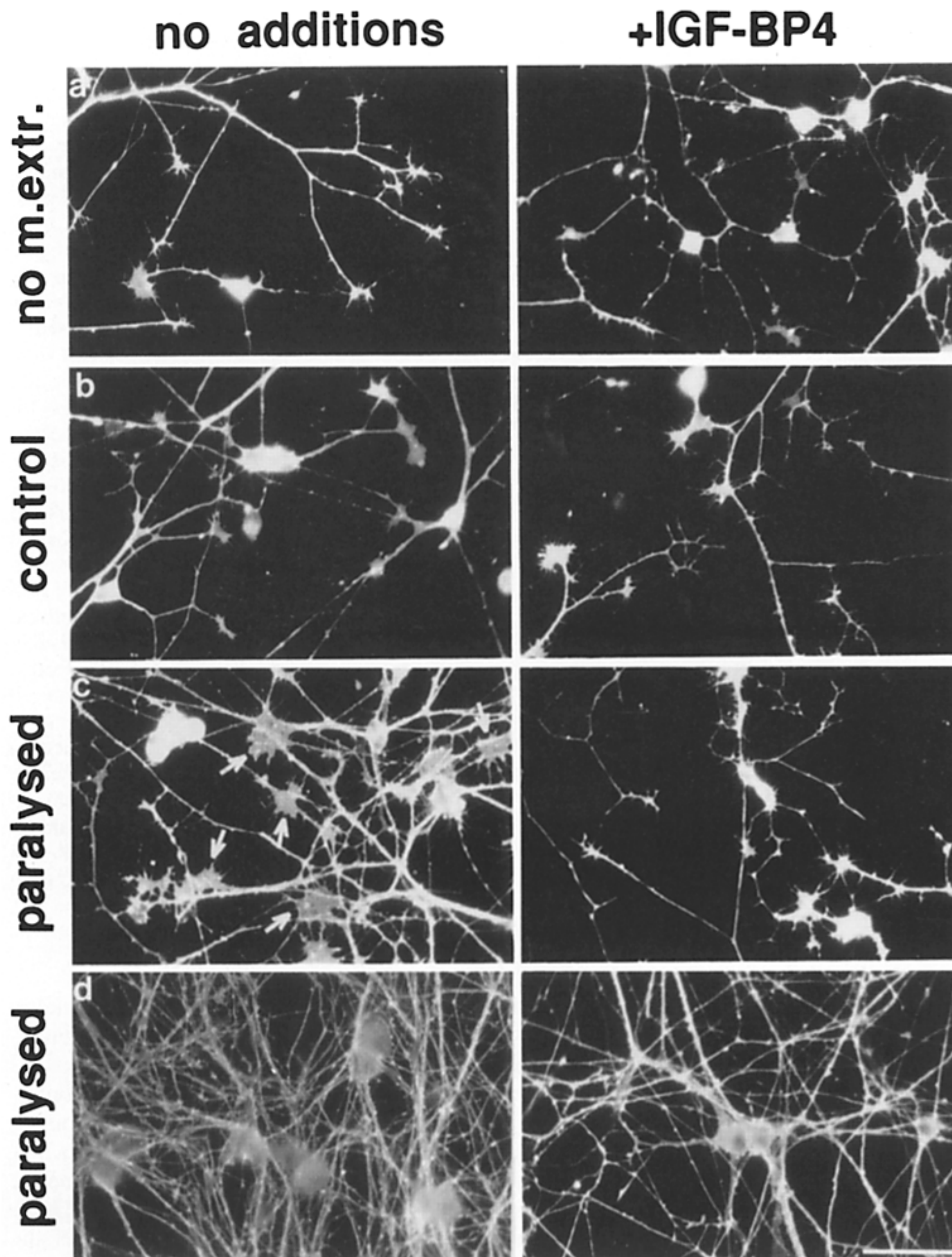

Figure 2. The effects of muscle extract from paralyzed muscle on neurite and growth cone morphology is suppressed by IGF-BP4. E8 chick DRG neurons were cultured for 20 h (*a–c*) and 3 d (*d*) in the absence (*left*) or in the presence (*right*) of 100 ng/ml of IGF-BP4. Photographs of indirect immunofluorescence labeling for the neuronal protein GAP-43 are shown in the figure. GAP-43 immunocytochemistry reliably visualizes neuritic morphology, including growth cones and filopodia (Allsopp and Moss, 1989; Widmer and Caroni, 1993). Other experimental conditions were as in Fig. 1. Additions to the culture medium: (*a*) no muscle extract; (*b*) 1 μg/ml of extract from control muscle; (*c* and *d*) 1 μg/ml of extract from paralyzed muscle. Note strikingly larger growth cones (*arrows* in *c*, *left*) in the presence of extract from paralyzed muscle, in the absence, but not in the presence of 100 ng/ml of IGF-BP4 (see Results section for quantitative analysis). Also note that the IGF-BP4 interfered with neuritic branching, but not with NGF-dependent DRG neuron survival in 3 d cultures (*d*). Bar, 45 μm.

derson et al., 1993). Chick motoneurons were cultured in the presence of CNTF and bFGF (Arakawa et al., 1990), whereas rat motoneurons were maintained with BDNF (Henderson et al., 1993). Therefore, under our experimental conditions, IGF-BP4 did not detectably interfere with the activity of NGF (Figs. 1 and 2), BDNF, or CNTF plus bFGF. These findings are further evidence for the high specificity of IGF-BP4 for IGF1 and IGF2.

In summary, our experiments demonstrate that the purified human recombinant IGF-BPs used in this study can be applied to specifically neutralize the neurite-promoting activities of purified IGFs and of complex protein extracts from paralyzed mouse muscle under primary culture conditions, suggesting that they may also block these activities when applied in excess in vivo.

IGF-BP4 Prevents Intramuscular Nerve Sprouting in Paralyzed Mouse Gluteus Muscle

To determine whether muscle IGFs are involved in the nerve sprouting reaction in paralyzed muscle we delivered purified human recombinant IGF-BP4 to mouse gluteus muscle that had been exposed to a paralyzing dose of Botulinum toxin A (Thesleff, 1989). The IGF-BP4 was released for 7 d at an estimated rate of 1 μg/day from an osmotic minipump implanted subcutaneously over the paralyzed muscle. Botulinum toxin A was applied shortly before implantation of the osmotic minipump. The dose of IGF-BP4 was selected by assuming a maximal muscle interstitial space concentration of IGFs of 100 ng/ml. This value is probably never reached in muscle, and it corresponds to the tissue content of IGFs found in the liver, i.e., the site with the highest contents of

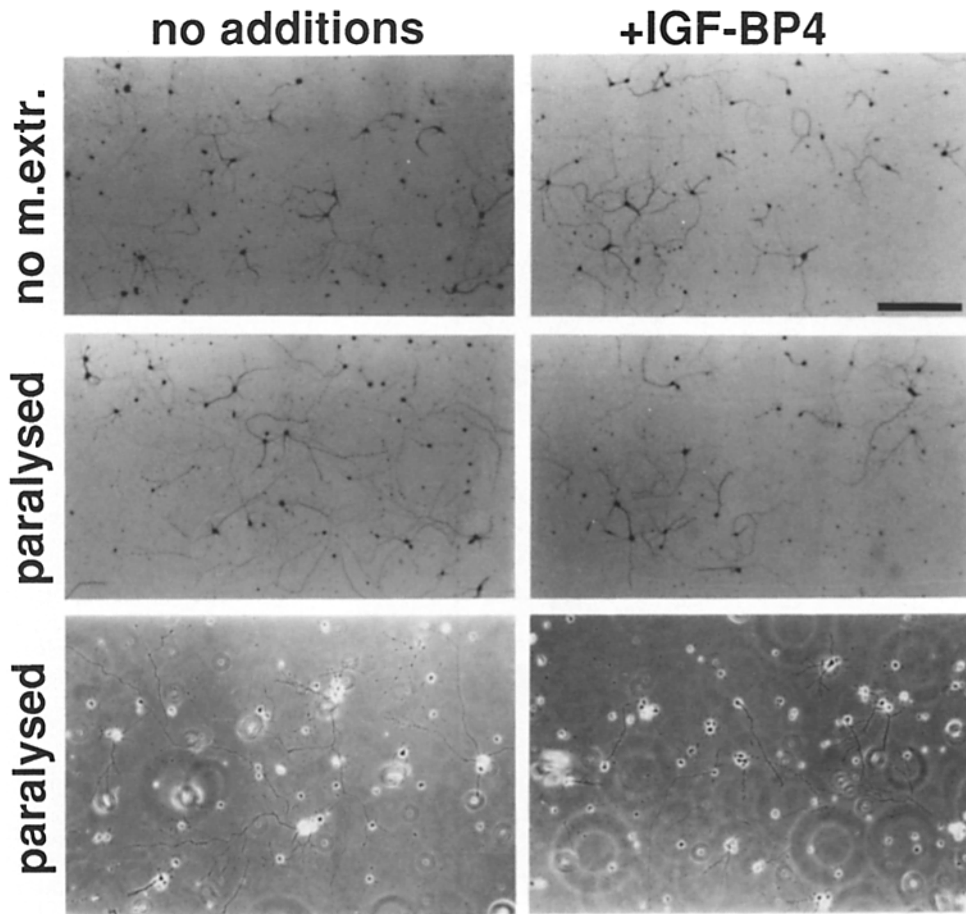

Figure 3. Suppression of the neurite-promoting activity in extracts from paralyzed muscle by IGF-BP4. Chick (*upper four panels*) or rat (*lower two panels*) spinal motoneurons were plated on a laminin-coated substratum in the presence of 2% horse serum and 2 ng/ml CNTF plus 10 ng/ml bFGF (*chick motoneurons*), or 2 ng/ml BDNF (*rat motoneurons*). Where indicated, 1 μg/ml of protein extract from paralyzed muscle and/or 100 ng/ml IGF-BP4 were also included in the culture medium. (*Chick motoneurons*) 2 d cultures; neurofilament-160 immunoreactivity visualized with alkaline phosphatase color reaction. (*Rat motoneurons*) 2 d cultures; phase contrast photographs of unfixed cells. Note effect of IGF-BP4 on neurite length in the presence of extract from paralyzed muscle. Also note lack of effect of IGF-BP4 on neuron numbers and on neurite length in the absence of muscle extract (see Table I for quantitative analysis). Bar, 400 μm (*upper four panels*); 200 μm (*lower two panels*).

IGFs in the body. The gluteus muscle system has proven to be particularly convenient in previous, similar studies (Brown, 1984; Caroni and Grandes, 1990; Gurney et al., 1992): it is superficial and only 4–5 muscle fibers across, thus facilitating the diffusion of agents from the subcutaneous space.

As shown in Fig. 4 *b*, and in agreement with previous reports (Holland and Brown, 1981), extensive nerve sprouting was detected in the gluteus muscle of animals 7 d after toxin application. Significantly, however, essentially no sprouts were detected in IGF-BP4-treated animals (Fig. 4, *c–e*). The figure also shows that subcutaneous application of long ^{3}R-IGF1 over the gluteus muscle of otherwise untreated animals also induced nerve sprouting, although less effectively than Botulinum toxin A. The most reliable effect of this IGF1 derivative with reduced binding to IGF-BPs, however, was a marked increase in the size and complexity of the terminal nerve branches at the neuromuscular junction (Fig. 3 *f*; estimated average endplate areas of 530 ± 149 μm^2 [control] and 1099 ± 251 μm^2 [with IGF1]). A quantitative analysis of the effects of IGF-BP4 on paralysis-induced sprouting is shown in Fig. 5. The results demonstrate that frequency and median length of the sprouts emerging from neuromuscular junctions of paralyzed muscles were dramatically reduced by IGF-BP4. In fact, sprouting values in the presence of the IGF-BP4 were only slightly higher than those detected in control non-paralyzed muscles (data not shown). The data also show that sprouting inhibition was due to the presence of the IGF-BP4 in the osmotic minipump, since in control experiments minipumps containing only carrier did not affect the sprouting reaction. In addition, when an osmotic minipump was applied over the contralateral non-paralyzed muscle, no interference with sprouting in the ipsilateral muscle was detected, arguing against a systemic mode of action of the IGF-BP4 in these experiments.

We did not test systematically the dose-response of IGF-BP4 in the sprouting experiment in vivo, because of the potential difficulties in the interpretation of the experiment due to in vivo diffusion of limiting amounts of binding protein. We did, however, notice that delivery of 100 ng of IGF-BP4 per day, i.e., a 10-fold lower dose significantly reduced the inhibitory effect on the sprouting response, indicating that the experimental conditions selected in our experiments were probably near to optimal. In conclusion, therefore, these experiments demonstrate that IGF-BP4 specifically suppresses the intramuscular nerve sprouting reaction in paralyzed mouse skeletal muscle, indicating that muscle IGFs are required for this process.

Discussion

We have shown that the neurite outgrowth-promoting activity of IGFs in vitro can be specifically blocked by the application of IGF-BP4. Using the same in vitro assay, we demonstrate that soluble protein extracts from paralyzed, but not from control adult mouse muscle contain substantial amounts

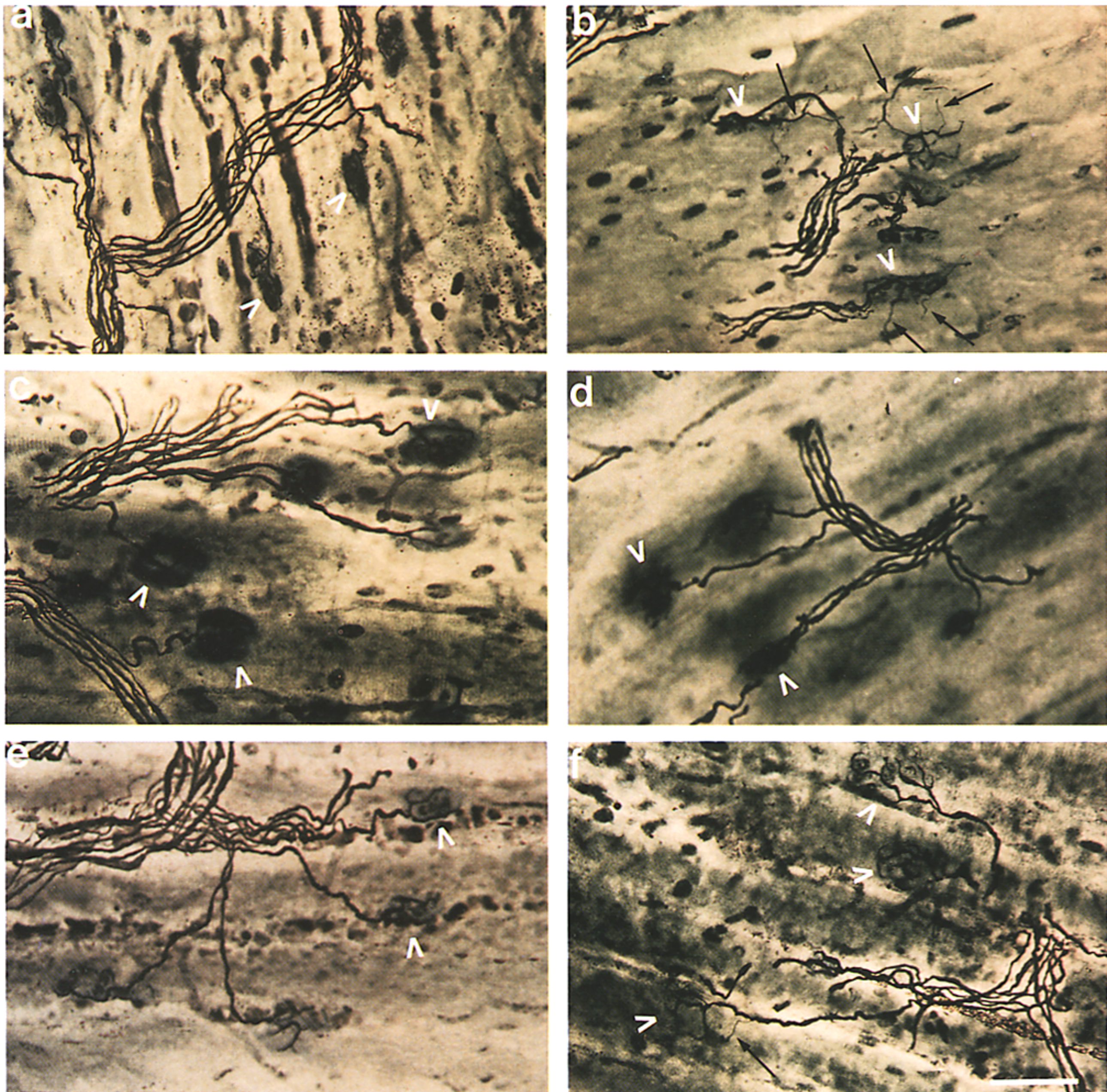

Figure 4. Suppression of terminal nerve sprouting in paralyzed mouse gluteus muscle in the presence of IGF-BP4. Combined silver esterase stainings of mouse gluteus muscle cryostat sections are shown in the figure. Muscle treatments: (*a*) control, untreated muscle; (*b–e*) 7 d after Botulinum toxin A-induced paralysis, with (*b*) and with (*c–e*) IGF-BP4; (*f*) daily local subcutaneous injections of 100 ng of long^{3}R-IGFl for 7 d. The white "*v*"-symbols point to the position of some of the neuromuscular junctions in the photographs; black arrows point to examples of sprouts in *b* and *f*. IGF-BP4 was delivered from an osmotic minipump implanted subcutaneously over the paralyzed muscle, as described in the Materials and Methods section. The estimated rate of delivery was ~1 μg of IGF-BP4 per day. Note presence of extensive terminal nerve sprouts in *b*, and absence of sprouting in the IGF-BP4-treated and paralyzed muscles in *c*, *d*, and *e*. Also note enlargement of the endplates (estimated average areas: 530 ± 149 μm^2 (*untreated*), 1099 ± 251 μm^2 [*IGFl-treated*]), presence of swellings in terminal nerve branches, and occasional sprouts in the presence of the IGFl derivative (*f*). Bar, 50 μm.

of IGF-BP4-sensitive neurite outgrowth-promoting activity. When applied in vivo, IGF-BP4 effectively suppressed intramuscular nerve sprouting in Botulinum toxin paralyzed mouse muscle. Our findings therefore indicate that muscle IGFs are required for nerve sprouting in paralyzed muscle. When combined to previous findings, our data support the notion that muscle IGFs are a central component of intercellular signaling in inactivated muscle.

Application of IGF-BPs to Block the Biological Activities of IGFs

IGF-BPs are valuable reagents to interfere with the activity of IGFs because they are naturally occurring IGF ligands with high affinity and specificity for these growth factors (Froesch et al., 1985; Baxter and Martin, 1989; Yang et al., 1989; Clemmons, 1990; Rechler and Nissley, 1990; Rosenfeld et al., 1990; Shimasaki and Ling, 1991). The association

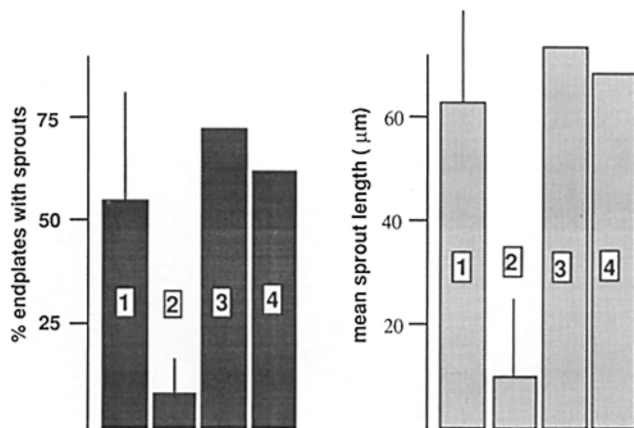

Figure 5. Suppression of terminal nerve sprouting in paralyzed mouse gluteus muscle by IGF-BP4. Percentage of endplates with sprouts (*left*) and mean sprout length values (*right*) are shown in the figure. Experimental conditions were as shown in Fig. 4. Animals were sacrificed 7 d after Botulinum toxin A-induced paralysis, and sprouting parameters were derived from silver esterase stained cryostat sections. Contents and site of implantation of osmotic minipump; (*1*) no minipump; (2) +IGF-BP4, over paralyzed muscle; (*3*) carrier, but no IGF-BP4, over paralyzed muscle; (*4*) +IGF-BP4, over contralateral non-paralyzed muscle. Data of experiments *1* and 2 are averages and standard deviations from five experimental animals (100 endplates per animal); experiments *3* and *4*: average values from two experimental animals each (50 endplates per animal).

constants of purified recombinant human IGF-BP4 and IGF-BP5 for human IGF1 and IGF2 are all approximately 2×10^{10} M^{-1} (Kiefer et al., 1992). Affinities for IGF1 and IGF2 are essentially undistinguishable, indicating that these IGF-BPs bind to shared sequences on the two IGFs. Such sequences are highly conserved in IGFs from man, mouse, and chick and represent ~70% of the total mature growth factor sequences (Humbel, 1990). For these reasons, human IGF-BP4 and IGF-BP5 probably bind with very similar affinities to IGFs from all these vertebrate species.

The in vitro association constants between the IGF-BPs used in this study and IGFs are 10–100-fold higher than those between the IGFs and the IGF1-receptor (the K_a of the IGF1-receptor for IGF1 is 6.7×10^8 M^{-1}; Steele-Perkins et al., 1988). This receptor tyrosine kinase probably is the main transducer of the biological activities of IGFs (Czech, 1989; Nissley and Lopaczynski, 1991). As a consequence, excess IGF-BP probably prevents extracellular IGFs from interaction with cell surface-associated IGF1-receptor, thus suppressing their biological activities. This prediction was verified in several biological assays involving mitogenic and differentiating effects of IGFs (Zapf et al., 1979; Kuoner and Smith, 1980; Mohan et al., 1989; Culouscou and Shoyab, 1991; Kiefer et al., 1992). It must, however, be pointed out that the opposite outcome, i.e., stimulation of IGF activity by IGF-BP1, −3, and −5 was also reported in a limited number of cases (Baxter and Martin, 1989; Clemmons, 1990). Stimulatory effects of IGF-BPs may require special experimental conditions to be detected, and may possibly involve binding of the IGF/IGF-BP complex to extracellular matrix. Thus, in a recent report, Clemmons et al. demonstrated that IGF-BP5 binds to cell surfaces and extracellular matrix components, and that such binding significantly reduces its affinity for IGFs (Jones et al., 1993). These authors therefore suggested that IGF-BP5 may serve as a delivery system to concentrate and release extracellular IGFs to the IGF1-receptor on cell surfaces. Overall, however, while IGF-BPs clearly prolong the half-life of IGFs, and may potentiate their actions under special conditions, it is clear that in most cases excess IGF-BPs in the extracellular space prevent the activation of IGF-receptors by IGFs.

While the effects of IGF-BPs on IGF activities in vitro have been studied extensively, sufficient amounts of purified recombinant IGF-BPs for in vivo experiments have only recently become available. In one report, intrabursal administration of IGF-BP3 in gonadotropin-treated immature female rats prevented follicle rupture, but not oocyte maturation (Bicsak et al., 1991). We recently found that the proliferation of muscle interstitial cells, one of the earliest events after muscle inactivation, is largely prevented by repeated local applications of IGF-BP5 (Caroni and Schneider, 1994). The experiments presented in this report demonstrate that local effects of IGF-BPs can be obtained in in vivo experiments extending over a period of a week. We noticed, however, that interference with intramuscular nerve sprouting was only effective when the IGF-BP was applied continuously with an osmotic minipump, and that daily applications had little inhibitory effect (Caroni, P., and C. Schneider, unpublished results). This may be due to a limited half-life of the IGF-BP in these in vivo experiments, although alternative explanations, e.g., rapid diffusion away from the site of application are also possible.

Our findings clearly indicate that the 26-kD IGF-BP4 did diffuse into the ipsilateral gluteus muscle, and that systemic diffusion was not sufficient to affect intramuscular nerve sprouting in the corresponding contralateral muscle. In addition, neurite outgrowth assays with muscle extracts from IGF-BP4-treated paralyzed muscle provide further evidence indicating that IGF-BP4 did diffuse into the gluteus muscle, where it bound to IGFs. When compared to those from paralyzed, but otherwise untreated muscles, such extracts were significantly less effective in inducing neurite outgrowth and growth cone spreading, suggesting that the IGF-BP4 applied in vivo reduced the fraction of active IGF in the paralyzed muscles (data not shown).

IGF-BP-3 and -5 are expressed in muscle (McCusker et al., 1989; Peter, M., and J. Zapf, unpublished results), and the expression of IGFs and their binding proteins is frequently coordinated and controlled by feedback mechanisms (Ceda et al., 1991; Neely and Rosenfeld, 1992; Lee et al., 1992; Conover et al., 1993). Therefore, it will be essential to obtain much more detailed information on the expression of IGF-BPs in the neuromuscular system, in order to formulate more precise hypotheses on the actions of the IGF system in neuromuscular development and regeneration. Furthermore, the recent discovery of specific proteolytic processes that affect the binding activity of the IGF-BPs introduces the exciting possibility that the actions of the IGFs may be modulated locally in the extracellular space (Campbell et al., 1992; Conover et al., 1993).

In conclusion, although the local biological roles of IGF-BPs may be complex and are presently not clear, our experiments indicate that they can be applied as pharmacological agents to interfere with the activity of IGFs in vitro and in vivo.

IGFs Play an Essential Role in Intramuscular Nerve Sprouting after Paralysis

Our experiments with IGF-BP4 in paralyzed mouse gluteus muscle clearly demonstrate that this protein specifically prevents intramuscular nerve sprouting, indicating that muscle IGFs are critically involved in this reaction. The experiments with extracts from paralyzed muscle suggest that IGFs are a major neurite promoting activity in these muscles. Therefore, when combined with previous observations on the time course of IGF induction in inactivated skeletal muscle fibers (Caroni and Schneider, 1994), and on the effects of IGFs in normal and impaired muscle (Caroni and Grandes, 1990; Lewis et al., 1993), our results strongly support the conclusion that muscle IGFs are major components of signaling in the inactivated neuromuscular system.

IGFs may promote nerve sprouting through the activation of IGF-receptors on motoneuron processes. Alternatively, activation of IGF-receptors on interstitial cells or the muscle fibers themselves may either be necessary or sufficient. Our experiments and those of Ishii et al. indicate that IGFs can act directly on sensory and motoneurons to promote neurite outgrowth in vitro (Recio-Pinto et al., 1986; Caroni and Grandes, 1990). In addition, we recently found that when applied subcutaneously to skeletal muscle in vivo, IGF1 accumulates at the neuromuscular junction (Caroni, 1993). This finding suggests that the neuromuscular junction may be an important site of action of muscle IGFs. Therefore, a direct mode of action of IGFs on motoneuron terminal branches to induce sprouting seems plausible. It is, however, likely that additional cellular reactions, some of them probably involving IGFs are also involved in bringing about the appropriate conditions for effective nerve sprouting (Pestronk and Drachman, 1978; Gatchalian et al., 1989; Sanes, 1989).

Our in vitro experiments suggest that IGFs are an important neurite outgrowth promoting component in paralyzed muscle. Thus, IGF1 and extract from paralyzed muscle both stimulated neurite outgrowth from dissociated DRG neurons in vitro in an IGF-BP4-sensitive manner. We demonstrated in a previous report that neurite outgrowth by cultured chick spinal motoneurons is strongly promoted by IGFs (Caroni and Grandes, 1990). Our experiments now demonstrate that protein extracts from paralyzed muscle contain IGF-BP4-sensitive neurite-promoting activity for chick and rat spinal motoneurons. In addition, 100 ng/ml of IGF-BP4 did not detectably affect the survival and neurite-promoting activities of NGF, BDNF, CNTF, or bFGF under our experimental conditions. These findings further confirm the high specificity of IGF-BP4 for IGF1 and IGF2, and strongly suggest that this IGF-BP interferes with intramuscular nerve sprouting in vivo by specifically interacting with IGFs.

IGFs may not be the only growth factors involved in the nerve sprouting reaction in inactivated muscle. Preliminary results suggest that the neurotrophin BDNF is upregulated in denervated muscle (Henderson, C. E., and A. Rosenthal, personal communication). In addition, potent stimulation of intramuscular nerve sprouting by local applications of ciliary neurotrophic factor was demonstrated (Gurney et al., 1992). Therefore, since a role for additional growth factors in the sprouting reaction can presently not be excluded, it is possible that although muscle IGFs are required for nerve sprouting, restorative reactions in inactivated muscle may be produced by multiple growth factors. According to this possibility, IGFs in inactivated muscle may promote reactions essential for sprouting, but additional factors may be required to induce a sufficient set of local changes to effectively promote and support intramuscular nerve sprouting. On the other hand, identification and elucidation of the possible roles of additional factors in nerve sprouting in inactivated muscle will require further in vivo perturbation studies.

Roles of IGFs and Their Binding Proteins in the Neuromuscular System

IGFs play important roles in muscle growth and differentiation, and they are the only growth factors known to specifically promote muscle differentiation (Florini et al., 1991). In addition, IGFs appear to be involved in multiple aspects of the development and regeneration of the neuromuscular system. Expression levels of IGFs during muscle development correlate with the extent of neuromuscular synaptogenesis, rather than with muscle growth or early differentiation (Ishii, 1989). Downregulation of muscle IGFs during development coincides with synapse elimination, and is abolished in Botulinum toxin paralyzed muscle (Ishii, 1989). Our findings now indicate that muscle IGFs are an essential component of signaling in inactivated adult rodent skeletal muscle. In addition, recent findings indicate that IGFs may be important components in promoting and accelerating peripheral nerve regeneration and functional recovery after lesion (Near et al., 1992; Lewis et al., 1993). It therefore appears that IGFs may play multiple and important roles in neuromuscular development and regeneration.

In conclusion, while more work is required to understand the role of IGFs in the neuromuscular system, it is clear from the results of this and other recent studies that IGFs play central roles in regenerative processes of the neuromuscular system. These findings may therefore have important implications for the study and treatment of pathologies of the neuromuscular system.

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