Levels of mRNA coding for motoneuron growth-promoting factors are increased in denervated muscle

(Xenopus oocyte/SC1 antibody/panning/tetrodotoxin-sensitive sodium channel)

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COMMUNICATION

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Communicated by Jean-Pierre Changeux, April 13, 1992

ABSTRACT Partial denervation of skeletal muscle induces sprouting of axons remaining within the muscle, possibly as a result of increased synthesis by denervated muscle fibers of motoneuron growth-promoting factors. Direct verification of this hypothesis has not been possible because the molecules responsible are not unambiguously characterized. We used Xenopus oocytes as a functional assay for mRNAs coding for secreted growth factors: preparations of mRNA from innervated and denervated neonatal muscle were injected into oocytes. Three days later, oocytes injected with denervated muscle mRNA expressed increased levels of nicotinic acetylcholine receptor and voltage-dependent sodium channels at their membrane. Proteins secreted by the same oocytes were tested for their effects on (i) neurite outgrowth from embryonic chicken ventral spinal cord neurons; (ii) survival in mixed culture of embryonic chicken motoneurons identified using the SC1 antibody; and (iii) survival of embryonic motoneurons purified by panning on SC1 antibody. In all three assays, media conditioned by oocytes injected with mRNA from denervated muscle contained significantly higher levels of biological activity than did those from oocytes injected with innervated muscle mRNA or water. mRNA was prepared from muscle at different times after denervation: a maximal increase was obtained already after 1 day, consistent with an involvement in sprouting. Synthesis of motoneuron growth-promoting factors is thus regulated by denervation in a parallel fashion to that of other key components of the neuromuscular junction.

The mature neuromuscular junction has a remarkable capacity for regeneration. For instance, not only does denervation induce increased levels of synthesis of acetylcholine receptor, N-cellular adhesion molecule, and other key proteins required for synaptogenesis (1-4), but regrowth of the presynaptic motoneuron is stimulated and directed with precision to the site of the original synapse (5-7). The mechanisms underlying this regeneration are of importance not only in understanding the plasticity of the neuromuscular system but also because similar mechanisms may be invoked during the initial development of the neuromuscular junction and, perhaps, of other peripheral and central synapses.

One of the best-defined systems for study of the plasticity of presynaptic elements is that of terminal and collateral sprouting of motor axons remaining within a partially denervated muscle (5, 6). In vivo evidence suggests that denervated muscle fibers release a "sprouting signal" that can diffuse limited distances within the muscle and induce new growth of established axons (5). The synthesis and/or release of this signal are probably under the control of electrical activity: direct stimulation of partially denervated muscle prevents sprouting (6). However, in the absence of a cDNA for the growth factor(s) responsible for the sprouting, the postsynaptic nature of the denervation-induced regulation is hard to prove.

As a first step toward this demonstration, several groups have reported that denervated muscle extracts contain higher levels of neurite- or survival-promoting activities for motoneurons or spinal neurons in vitro (8-12) and in vivo (13) than do extracts of innervated muscle. However, several considerations make it difficult to extrapolate these results to the in vivo situation. (i) It is possible that the observed increases do not reflect a change in the rate of synthesis or secretion of the active factors but simply reflect local accumulation of proteins that would normally have been transported in a retrograde manner by motor axons from muscle to the spinal cord.

(ii) A second factor hindering reliable evaluation of the growth-promoting activity released by a given muscle is that preparation of tissue extracts releases many soluble components that do not normally come into contact with the motoneuron in situ. Any growth factor normally sequestered within the muscle or any muscle component that has an artificial growth-promoting activity for motoneurons in the different test systems may, therefore, contribute to an artificially high estimate of the neurotrophic support received by the motoneuron from the muscle. Conversely, inhibitors released by homogenization may prevent the true activity from being detected.

In the light of these problems, we sought to define a system that would allow measurement of the synthesis and secretion by muscle of molecules that promote motoneuron growth and survival. We turned to Xenopus oocytes, whose highly efficient translation machinery has often been used in the study and cloning of membrane proteins (14, 15). By injecting preparations of mRNA characteristic of innervated and denervated muscles, we hoped that oocytes would process and secrete exogenous proteins bearing appropriate signal sequences; these proteins could then be assayed independently of those retained within the cell.

(iii) The third objection to many of the studies comparing innervated and denervated muscle is that the motoneurons were imperfectly identified or studied in the presence of other cells. For this reason, we studied the effects of proteins secreted and synthesized by the injected oocytes on the survival of pure motoneurons in low-density culture. The motoneurons were purified by panning (16) using the SC1 monoclonal antibody, which, within the 5-day embryonic chicken spinal cord, labels only motoneurons and floor-plate cells (17). The latter may be removed by micro-dissection.

Abbreviations: IGF-1 and IGF-2, insulin-like growth factor 1 and 2, respectively.

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panning then yields near-homogeneous cultures, containing routinely >95% SCI-positive neurons. To cover as wide a spectrum as possible of motoneuron growth-promoting activities, however, two other activities of the secreted proteins were also monitored: (i) neurite outgrowth from early ventral spinal neurons and (ii) survival of identified motoneurons in the presence of other cells of the spinal cord. The results from all three bioassays were consistent and point to the existence of denervation-induced regulation of the synthesis and secretion of proteins with properties similar to those expected for the hypothetical "motoneuron growth factor".

METHODS

Neurite Outgrowth Assay. Ventral halves of spinal cords were dissected from 4.5-day Leghorn chicken embryos (Hamburger and Hamilton stage 24–25, ref. 18), treated with trypsin, and dissociated as described (9). The cells were plated in uncoated Nunc 24-well dishes at a density of 20,000 cells per well in 400 µl of complete serum-free medium (Ham’s F-12 supplemented with penicillin (100 international units per ml), streptomycin (100 µg/ml), glucose (10 mM), and insulin (5 µg/ml)). Neurite outgrowth was counted by using phase contrast and expressed as the percentage of surviving cells with at least one neurite greater than two cell diameters in length.

Survival Assay for Identified Motoneurons in Mixed Culture. Glass coverslips were coated with poly(l-ornithine) (1.5 µg/ml in water) and laminin (3 µg/ml in F-12 medium). Total spinal cord cells were seeded on coverslips at 20,000 cells per 16-mm well. After incubation with serial dilutions of oocyte-conditioned medium for 3 days, cultures were fixed and stained with the SC1 antibody (16). Motoneurons labeled with SC1 were visualized in the fluorescence microscope; their number in at least 20 microscopic fields per coverslip was expressed as a percentage of total neurons visualized by using Nomarski optics or background fluorescence in the same fields. Floor-plate cells, identified as SC1-positive flat cells with neither a rounded cell body nor neurites, were omitted from both counts.

Survival of Purified Motoneurons in Terasaki Dishes. Spinal cords of 4.5-day embryos were subdissected by using microscissors to remove the floor-plate. After dissociation as above, the resulting cell suspension was incubated for 1 hr in a Petri dish containing immobilized SC1 antibody, as described (16). At the end of this period, unbound cells were eliminated by washing, and bound cells were subsequently eluted with an excess of SC1 antibody. Motoneurons thus purified were seeded in Terasaki wells (in 10 µl of medium) on laminin in the presence of serial dilutions of oocyte-conditioned medium. Each dilution was tested in at least six independent wells. Culture medium was L15, containing sodium bicarbonate (0.625 mg/ml), insulin (5 µg/ml), putrescine (0.1 mM), conalbumin (0.1 mg/ml), sodium selenite (30 nM), progesterone (20 nM), glucose (20 mM), penicillin (100 international units per ml), and streptomycin (100 µg/ml). After 3 hr, all phase-bright cells in each well were counted to obtain the 100% survival value. In subsequent daily measurements, only those phase-bright cells with neurites greater than two cell diameters in length were taken into account.

Preparation of mRNA. Poly(A)^+ RNA was prepared from fresh or frozen 6-day neonatal chicken thigh muscle, containing mainly slow postural fibers, by using the method of Chomczynski and Sacchi (19) followed by oligo(dT)-cellulose chromatography, or using the Fast-Track kit (Invitrogen).

Oocyte Injection and Electrophysiology. All operations were done in the sterile hood. Oocytes from the same female were injected with 50 nl of RNA at 2 mg/ml (25 oocytes per RNA preparation) and maintained at 20°C in 3.5 ml of Barth’s medium (88 mM NaCl/1 mM KCl/0.8 mM MgSO4/0.4 mM CaCl2/2.4 mM NaHCO3/15 mM Hepes) supplemented with gentamicin. One day later, the medium was replaced with fresh Barth’s medium, and the incubation was continued for a further 48 hr. After harvesting conditioned medium (see below), oocytes were voltage-clamped at −80 mV by using a two-microelectrode voltage-clamp amplifier. ND96 (96 mM NaCl/2 mM KCl/1 mM MgCl2/1.8 mM CaCl2/5 mM Hepes, pH 7.5) continuously bathed the oocyte in the recording chamber. Carbachol (100 µM) was applied directly in the bath, with the perfusion stopped.

Preparation of Oocyte-Conditioned Media. At the end of the second incubation (48 hr), secreted proteins from 25 oocytes in 3.5 ml were dialyzed overnight at 4°C against Ham’s F-12 medium before assay, using Spectro-pur-2 tubing (exclusion limit, 12–14 kDa) that had been autoclaved and presaturated with sterile bovine serum albumin (2% in F-12 medium). Sterile filtration of samples was not done, to avoid potential losses at these low protein concentrations. Preliminary experiments (data not shown) demonstrated that only low levels of biological activity were detected in the medium after shorter incubations, and that no improvement was obtained by incubating longer than 2–3 days. Conditioned media were routinely assayed immediately but retained their activity after several weeks at −20°C.

RESULTS

Levels of mRNA for Nicotinic Acetylcholine Receptor and Sodium Channels in Denervated Muscle. mRNA levels for the α subunit of the acetylcholine receptor are known to be up-regulated in denervated chicken muscle at neonatal stages (1, 20). We used this observation to verify the capacity of the Xenopus oocyte system to reproduce functional effects of muscle denervation. The sciatic nerve on one side of anesthetized 6-day-old chicks was sectioned. Three days later, the chicks were sacrificed, and poly(A)^+ RNA was prepared from denervated leg muscles after removal of nerve branches. Contralateral leg muscles were used as innervated controls. Yields of poly(A)^+ RNA were similar from control and denervated muscle (typically 40 µg per g of wet weight). Each preparation of RNA was injected as described in Methods. Currents corresponding to the nicotinic acetylcholine receptor were increased ≈2-fold in oocytes injected with denervated muscle mRNA (Fig. 1A), as expected if the mRNA was representative of that in the muscle (20, 21). Increased expression of acetylcholine receptor using denervated muscle mRNA was, therefore, used as an internal control in the experiments described below; batches of oocytes failing to show this regulation, either because they expressed all messengers poorly or because mRNA degradation had occurred, were not taken into consideration.

Voltage-activated sodium currents recorded after injection of mRNA from denervated muscle were also significantly higher than those obtained with innervated muscle mRNA (Fig. 1B). The increase was harder to quantify because sodium currents were not detectable in some mRNA preparations from innervated muscle; it was at least 4-fold. Similar observations have been made on denervated rat muscle; expression of the tetrodotoxin-insensitive channel is strongly up-regulated (refs. 22–24, but see ref. 21). Chicken muscle, however, is reported not to express tetrodotoxin-insensitive channels (25); in accordance with this, the sodium currents we observed were completely inhibited by 300 nM tetrodotoxin (Fig. 1B).

Effects of Proteins Secreted by Oocytes on Neurite Outgrowth from Ventral Spinal Neurons. The growth-promoting properties of oocyte-conditioned media were tested in three assays: (i) neurite outgrowth from embryonic ventral spinal neurons; (ii) survival of identified motoneurons in mixed
cultures of ventral spinal cord; and (iii) survival of motoneurons purified by panning on SC1 monoclonal antibody.

The first assay was most directly related to the sprouting observed in vivo and had previously been used to demonstrate an increase in neurite-promoting activity of muscle extracts after denervation (9). Ventral spinal cords were dissected from 4.5-day chicken embryos, dissociated, and plated in uncoated plastic dishes in serum-free medium. In independent experiments (data not shown), such cultures were shown to contain ~50% cells stained by the SC1 antibody (presumptive motoneurons), and by this criterion the majority of cells extending neurites were motoneurons. Proteins secreted by oocytes injected with mRNA from control innervated muscle or with the same volume of water, or proteins secreted by uninjected oocytes (data not shown), had no detectable activity in the neurite-outgrowth assay at any concentrations tested (Fig. 2A). In marked contrast, medium conditioned by oocytes injected with mRNA from denervated muscle clearly enhanced neurite outgrowth from a subpopulation of ventral spinal neurons, probably motoneurons (see above). Higher concentrations of conditioned medium than those shown could not be tested, as oocyte products had cytotoxic effects on all cells. The neurite-promoting activity of oocyte-conditioned medium was completely destroyed by trypsin (data not shown) and quantitatively retained after dialysis (exclusion limit, 12–14 kDa).

Uninjected oocytes that were maintained in depolarizing conditions (150 mM K+) did not secrete biological activity; the observed increase using denervated muscle mRNA is, therefore, unlikely to be mediated indirectly via changes in oocyte membrane potential. Proteins secreted after injection of innervated muscle mRNA did not inhibit the activity of concomitantly added muscle extract, suggesting that the lack of biological activity in these conditioned media resulted from the absence of a growth-promoting activity, rather than from the presence of an inhibitor. Batches of oocytes that failed to show increases in nicotinic acetylcholine receptor never produced increased levels of neurite-promoting activity.

Effects of Oocyte-Conditioned Medium on Survival of Identified Motoneurons in Mixed Culture. It is possible that the "sprouting signal" whose presence is induced from in vivo observations may belong to the class of neurotrophic factors, one of whose principal roles is to permit survival of embryonic neurons (26). For this reason, we also tested the effects of oocyte-conditioned medium on survival of identified motoneurons in the presence of the other cells of the spinal cord (Fig. 2B). The method used was based on the recently developed technology involving the SC1 monoclonal antibody, which labels motoneurons and floor-plate cells in early embryonic chicken spinal cord (16, 17). Cultures of total spinal cord from 4.5-day chicken embryos were performed on...
laminin-coated coverslips. In these conditions motoneurons, but not other spinal neurons, die within 3-4 days in the absence of supplements, such as muscle extract (16). After incubation with indicated dilutions of oocyte-conditioned medium for 3 days, cultures were fixed and stained with the SCI antibody. The number of surviving motoneurons in the fluorescence microscope was expressed as a percentage of total neurons in the same fields. Injection of mRNA from denervated muscle led to secretion of significantly higher levels of survival-promoting activity than did injection of innervated muscle mRNA or water (Fig. 2B). The survival effect was selective for motoneurons: in 20 microscopic fields, the number of SCI-negative neurons after 3-day culture with medium conditioned by oocytes injected with denervated muscle mRNA was 246 ± 78 (mean ± SEM; n = 6), and with innervated muscle mRNA was 251 ± 54 (n = 5).

Survival-Promoting Activity of Secreted Proteins for Purified Motoneurons. Motoneurons were purified by subdissection of the spinal cord to remove floor-plate and subsequent panning on dishes coated with SCI antibody (16). The cells were seeded in Terasaki wells on laminin in the presence of the indicated concentrations of oocyte-conditioned medium (Fig. 2C). Their purity (>95%) was confirmed by labeling of proliferative cultures with SCI antibody. Survival was quantified after 3 hr to obtain the 100% survival value and subsequently daily by phase-contrast microscopy; results from 1 day in culture are shown. Whereas proteins secreted by oocytes injected with water or with innervated muscle mRNA showed only weak survival-promoting activity, two completely independent preparations of mRNA from denervated muscle provided significant neurotrophic support (up to 37% of initially seeded motoneurons). After the same period in this experiment, optimal concentrations of muscle extract gave 48% survival. In other experiments (data not shown), oocyte-conditioned medium could support motoneuron survival for at least 3 days.

Motoneuron Survival-Promoting Activity as a Function of Time After Denervation. In partially denervated adult muscle, the first sprouts appear after 2-3 days, implying that the up-regulation of synthesis of the sprouting signal, if any, should occur at early times post-denervation (5). We assayed the in vitro survival-promoting activity of oocytes injected with mRNA prepared from denervated and control muscle at different times after the operation (Fig. 3). Secreted proteins were tested by using both purified motoneurons (Fig. 3A) and identified motoneurons in mixed cultures (Fig. 3B). At the earliest time tested (1 day after denervation), a clear increase was already apparent in both assays. Indeed, using purified motoneurons we detected no further increase in messenger levels during the following days (Fig. 3A). However, it is possible that oocyte synthesis or secretion machinery was saturated at high levels of the corresponding mRNAs, hiding subsequent changes in mRNA abundance.

DISCUSSION
The results presented here strongly suggest that levels of mRNA coding for molecule(s) with properties similar to those expected for a motoneuron neurotrophic factor are upregulated after denervation of skeletal muscle. Whereas mRNA from innervated muscle induced secretion of only low levels of biological activity by oocytes, injection of denervated muscle mRNA resulted in the accumulation in the oocyte culture medium of trypsin-sensitive macromolecules that enhanced both survival of purified and identified motoneurons and neurite outgrowth from a population of ventral spinal neurons enriched for motoneurons. The increase in mRNA levels was apparent as early as 24 hr after denervation and, therefore, potentially rapid enough to underlay both the in vitro phenomenon of sprouting (5) and the denervation-related increase in in vitro neurite-promoting activity of neonatal muscle extracts (8-13).

The bioassays chosen monitored different aspects of motoneuron development. Neurite outgrowth is, perhaps, the closest in vitro equivalent to motor nerve sprouting, although significant differences may exist both at the level of the differentiation state of the motoneurons and of the substrate upon which outgrowth occurs. The other assays measured neuronal survival in different contexts. Neonatal motoneurons die rapidly after axotomy, presumably as a consequence of deprivation of muscle-derived growth factors (27, 28), which cannot at this stage be supplied by the peripheral nerve (29). The up-regulation of mRNA for survival-promoting activity that we observe may limit motoneuron death when axons are severed at short distances from the muscle; it is consistent with previous observations on extracts of similar denervated preparations (11, 13). Of the three assays, the only one to show any significant activity using innervated muscle mRNA was the survival of motoneurons in mixed culture. Although this assay has the advantage that the motoneuron grows in a cellular environment more close to that in vivo, it should be noted that the non-motoneurons in these cultures are directly exposed to peripherally derived molecules from which they would normally be shielded. It is
conceivable that the effect of innervated muscle mRNA is an indirect one in this system, artificially mediated by other cells of the spinal cord.

Our aim was to study the effects of denervation itself; we thus cannot exclude the possibility that the increases observed were due to nerve degeneration products rather than muscle activity, nor can a contribution of non-muscle cells—e.g., Schwann cells—be ruled out, although care was taken to remove nerve segments from the muscle used. The biological activity observed was not that of ciliary neurotrophic factor (CNTF) or nerve growth factor (NGF) which, although they are known to be present in lesioned peripheral nerve (29, 30), are inactive on motoneuron survival and neurite outgrowth in these serum-free culture conditions (16). The same argument eliminates basic fibroblast growth factor (bFGF) as a candidate muscle-derived factor (16). The effects of cholera acetyltransferase development factor (CDF; ref. 31) in this system, and its regulation after denervation are unknown.

We considered the possibility that the increase in growth-promoting activity after denervation might be the result of synthesis of insulin-like growth factors (IGF-1 and -2). mRNA for IGF-2 is up-regulated in muscle 2- to 4-fold after denervation in 2-week-old rats (32), and IGF-1 mRNA is induced even more rapidly (33). Furthermore, both IGFs induce sprouting when applied exogenously to rodent muscle in vivo, or in vitro, to embryonic chicken motoneurons, which possess binding sites for IGFs at their membrane (34). These growth factors are, therefore, attractive candidates for the role of sprouting signal. However, the concentration of insulin (1 μM) in our basal culture medium is close to the Kd of insulin for the type 1 IGF receptor (35) and 50-fold higher than the concentration reported to be required for half-maximal stimulation of neurite outgrowth from embryonic motoneurons in vitro (34). Furthermore, IGF-1 and IGF-2 are without significant effect in vitro on motoneuron survival (34, 36) and do not affect neurite outgrowth in these culture conditions (C.E.H., unpublished results).

Another interesting possibility is that the protein whose mRNA was up-regulated by denervation is, in fact, involved in secretion of the growth factor, while synthesis of the growth factors is constant. This hypothesis was made tentatively to account for both in vivo sprouting and our in vitro observations. It is made less probable in our experiments, although not excluded, by the observation that in dishes in which one or more oocytes spontaneously lysed toward the end of the culture period, biological activity was always considerably lower than normal values.

This oocyte-based strategy has advantages over the usual procedure of direct assay of tissue homogenates to follow in vivo changes. For example, using oocyte injection only those molecules that are normally secreted are subjected to assay. This result may explain why mRNA from innervated neonatal muscle gave only a low signal in the oocyte assay, whereas tissue extracts of denervated muscle and many other tissues unrelated to the motoneuron contain easily detectable levels of motoneuron neurite- and survival-promoting activities (37). The former result seems more likely to reflect the true state of muscle–motoneuron interactions at a stage when both naturally occurring cell death and synaptogenesis are essentially complete. We previously showed that when conditioned media, as opposed to extracts, were prepared from cultures of different embryonic tissues, many cell types secreted only low levels of neurite-promoting activity for spinal neurons, whereas muscle produced high levels (38). The oocyte translation system may be seen as an alternative to this approach for secreted proteins in cases such as mature innervated muscle, for which representative cell cultures are not readily available.

A further advantage of this technique is that, in the absence of a well-characterized cDNA for a motoneuron growth factor, it provides the only means of determining whether increases in neurotrophic activity after denervation result from increased synthesis or simply from local accumulation in the absence of motor axons. These results strongly support the former hypothesis and should allow us to determine whether this regulation is activity-related or induced by nerve degeneration. Finally, our observations raise the possibility of direct expression cloning of these and other unidentified secreted factors.

We thank Clément Mettling and Joël Nargeot for many helpful discussions and A. Prochiantz, D. Thierry-Mieg, and J. Valmier for comments on the manuscript. This work was supported by the Institut de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies (A.F.M.), and the Institut pour la Recherche sur la Moelle Épinière. F.A.R. received studentships from the Ministère de la Recherche et de la Technologie and the A.F.M.