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The Effect of $ACTH_{4-10}$ on Protein Synthesis, Actin and Tubulin During Regeneration

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EDWARDS, P. M., J. VERHAAGEN, T. SPIERINGS, P. SCHOTMAN, F. G. I. JENNEKENS AND W. H. GISPEN. *The effect of $ACTH_{4-10}$ on protein synthesis, actin and tubulin during regeneration.* BRAIN RES BULL **15**(3) 267–272, 1985.—The effects of $ACTH_{4-10}$, a peptide fragment of corticotropin, on rat dorsal root ganglia (DRG), spinal cord and sciatic nerve were studied following a crush lesion of the sciatic nerve. The *in vitro* total protein synthesis rate of DRG L4, L5 and L6, measured one and three days after ipsilateral nerve crush, were not altered by various $ACTH_{4-10}$ treatment regimes. Likewise, neither $ACTH_{4-10}$ treatment of sham-operated rats nor *in vitro* exposure of control ganglia to peptide, resulted in changes in synthesis rate. Four days after crush lesion, the amounts of actin and tubulin in the ventral horn L2–L5 region of the spinal cord and of actin in DRG L5 were estimated following 2-dimensional separation. No significant effect of ACTH treatment was found. Degeneration-associated changes in the protein profiles of segments of sciatic nerve were not altered by $ACTH_{4-10}$ treatment. The data are discussed in relation to the possible site of action of neurotrophic ACTH-like peptides.

Neurotrophic peptides ACTH Rat sciatic nerve Regeneration Protein synthesis Cytoskeletal proteins

CORTICOTROPIN (ACTH) and melanocyte-stimulating hormone (MSH) are derived from a common precursor polypeptide (POMC) and were originally discovered as pituitary hormones. In recent years, these peptides have been shown to be also present in nerve cells [23] and to modulate neuronal function [11]. The neurotrophic properties of ACTH-related peptides have been demonstrated by their ability to facilitate the regeneration of peripheral nerves [2, 4, 27]. The beneficial effects of ACTH on nerve tissue appear to be independent of evoked adrenocortical secretions. This is exemplified by the observation that $ACTH_{4-10}$, which has little adrenocortical-stimulating effect, is as active as $ACTH_{1-24}$ in facilitating the repair process [2].

A neurotrophic role for ACTH peptides had previously been proposed following the demonstration of their ability to modulate macromolecular synthesis in various neural systems [13]. Changes in RNA and protein metabolism form part of the cell body response to axonal injury [16], therefore it was plausible that the facilitatory effects of ACTH on nerve repair are mediated by an effect on protein synthesis. Studies on spinal cord extracts [5] indicated a stimulatory effect of $ACTH_{4-10}$ on total protein synthesis in unlesioned rats. However, neither crush lesion of the sciatic nerve, nor systemic treatment of lesioned rats with $ACTH_{4-10}$ produced changes in the protein synthesis of extracts. In this paper, we report analogous studies carried out on intact DRG. Compared with spinal cord extracts, DRG offer two advantages: the measured synthetic activity derives mainly from cells directly involved in the response to sciatic nerve injury and the rate of protein synthesis is closer to that measured in nerve tissue *in vivo* [12].

Axoplasmic transport plays a crucial role in the cellular responses to axon injury, since it serves as the means of relaying signals to the cell body and of supplying the axon with materials essential for growth. The rate of outgrowth in several instances correlates with the slow phase of axoplasmic transport [8] and changes in specific components of the rapid phase have been reported [7,20]. Rapid axoplasmic transport can only occur in the presence of an intact microtubular system, which together with actin and other cytoskeletal proteins, is supplied by the slow phases of transport [19]. However, studies on changes in axoplasmic flow and cytoskeletal proteins during regeneration have not given clear indications as to the significance of these factors in limiting regeneration. A preliminary study, on a small number of animals, suggested that the spinal cord content of actin and tubulin might be increased in response to $ACTH_{4-10}$ treatment [1]. We describe here a more extensive

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study on the effects of treatment with $ACTH_{4-10}$ on the content of α- and β-tubulin and actin in the spinal cord in rats recovering from a crush lesion of the sciatic nerve, using essentially the same protocol as was used by Bijlsma [1]. The Coomassie Blue staining method was chosen in order to obtain the best quantitative data for actin and tubulin, which are major protein components of the spinal cord. Minor protein components, such as can be detected with the more sensitive but less quantitative silver methods, were therefore not studied. Studies on the protein profiles in DRG and sciatic nerves are also described.

Studies on the critical period during which ACTH-like peptides exert a beneficial effect on recovery have indicated an action on processes occurring early in the response to injury [15]. Furthermore, histological investigations have shown that ACTH treatment increases the number of regenerating sprouts and this effect is most marked at early times [3]. The results of the studies presented in this paper are discussed in relation to the possibility that ACTH alters the initial sprouting response to injury [15].

METHOD

Animals, Surgery and Treatment

Female rats (120–140 g) of an inbred Wistar strain (TNO, Zeist, NL) were randomised over experimental groups prior to surgery. Crush lesions of the sciatic nerve and sham operations were performed as described previously [2]. The proximal boundary of the lesion was marked by an epineural suture [3]. Immediately after surgery and every 48 hr subsequently, rats were injected subcutaneously with either 0.5 ml saline or 10 μg $ACTH_{4-10}$ (Organon Int. BV, Oss, NL) in saline. In experiments in which only one dose of $ACTH_{4-10}$ was administered, this dose was delayed until 24 hr after surgery. All rats were killed 90 min after the last dose of $ACTH_{4-10}$. All experiments for measurement of individual proteins were carried out blind, the code indicating the treatment group for each sample being broken after the results of the scanning had been obtained.

Measurement of Total Protein Synthesis in Dorsal Root Ganglia (DRG)

Lumbar DRG, L4, L5 and L6, were dissected out and rinsed in incubation buffer (25 mM HEPES, 22 mM NaOH, 118 mM NaCl, 4.4 mM KCl, 1.5 mM K_2HPO_4, 1.3 mM $MgSO_4$, 2.6 mM $CaCl_2$, 12 mM glucose, pH 7.6) gassed with O_2/CO_2 (95:5) at room temperature. The ganglia were placed in tubes containing 0.5 ml medium at 37°C and preincubated for 30 min. The medium was replaced with a fresh 0.5 ml medium to which L-[1-^{14}C]-leucine (1 mM, 0.6 μCi/μmole, The Radiochemical Center, Amersham, UK) was added and the incubation carried out at 37°C for 1 hr. Throughout incubation and preincubation, the tubes were shaken gently and O_2/CO_2 was bubbled through the medium. In experiments on the effect of $ACTH_{4-10}$ *in vitro*, intact rats were used. In order to increase the time of exposure to peptides *in vitro*, $ACTH_{4-10}$ was present in both preincubation and incubation media and the incubation was continued for 6 hr. Incubation was stopped by transferring the tubes to ice-water. The ganglia were washed successively in 1 ml of solution containing 1 mM leucine and TCA as follows: 10% TCA (ice-cold, 10 min), 5% TCA (90°C, 15 min), 5% TCA (room temperature, 2×10 min) then, at room temperature for 20 min, successively in 2 ml methanol, methanol/chloroform (1:1), ether. Final traces of ether were removed under an air stream and the ganglia weighed, dissolved in 0.5 ml Lumasolve (Baker, Deventer, NL), then water (100 μl) and Beta-count (4 ml, Baker, Deventer, NL) were added prior to scintillation counting. The specific activity of leucine in the precursor pool was assumed to be the same as that added for calculation of the incorporation. Incorporation was linear for at least 7 hr. While there was essentially no difference between individual rats, left and right sides or L4, L5 and L6 ganglia, the experiments on *in vitro* peptide effects were designed to divide these variables as evenly as possible over the treatment groups. The largest experimental variable was the dry weight of individual ganglia, which was probably due to inconsistencies in dissection and was inversely correlated with incorporation rate. The inverse correlation may be related to inclusion of less active material (e.g., nerve fibres) or diffusional restrictions in larger ganglia.

Measurement of Cytoskeletal Proteins in Spinal Cord

The ventral horn region of the lumbar (L2–L5) spinal cord was dissected out as described by Bijlsma *et al.* [5]. The tissue was homogenised in buffer (0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, 2 mM $MgCl_2$, pH 7.6; tissue/buffer=1:4, w/v) by 5 up-and-down passes of a Potter-Elvehjem teflon/glass homogeniser (clearance 0.125 mm, rotating at 700 rpm). The homogenate was centrifuged at 1000 g for 10 min and the supernatant processed for gel electrophoresis. Samples, containing 30 μg protein, were mixed with denaturant as described by Burke and DeLorenzo [10] to give (final concentrations) 0.25% SDS, 4.5 M urea, 2% TX-100 and ampholines, 0.4% pH 3.5–10 and 1.6% pH 5–7 (LKB, Zoetermeer, NL). Separation of proteins in the first (pH-dependent) and second (SDS) dimension were further carried out as described by Zwiers *et al.* [29]. The gels were fixed, stained with Fast Green, photographed under standard conditions and contact prints prepared. The prints were scanned with a Zeiss cytospectrophotometer coupled to a Hewlett-Packard computer, to obtain an integrated value for the density and area of individual spots.

Measurement of Cytoskeletal Proteins in DRG

The procedure for DRG was altered from that used for spinal cord (see above and [1]) in order to obtain a more complete extraction of cellular proteins and to obtain a better separation of proteins in the region of the IEF gel where actin and tubulin migrate. L5 DRG were carefully dissected free from ventral roots then frozen in liquid nitrogen and crushed by use of a stainless-steel pestle and mortar cooled in liquid nitrogen. Each ganglion was then extracted at room temperature with 8 μl of 1.0% SDS, 10% β-mercaptoethanol, 8 M urea, in a tube held for 1 min in a sonic cleaning bath. After standing for 30 min, the extract was diluted to give final concentrations of 0.33% SDS, 6% β-mercaptoethanol, 7.5 M urea, 2% TX-100, 1.6% pH 5–7 and 0.4% pH 3.5–10 ampholines in 24 μl total volume. The extract was sonicated again for 1 min and then left to stand for 30 min. Undissolved material was removed by centrifugation at 100,000 g for 1 hr in a Beckman airfuge. The proteins present in 3 μl of the supernatant were separated on IEF gels in capillary tubes, followed by slab SDS-polyacrylamide electrophoresis in thin (0.6 mm) gels cast on GelBond (3M Co., Leiden, NL). The methods were similar to those described by Neukirchen *et al.* [22], modified as described by Edwards *et al.* [14]. The

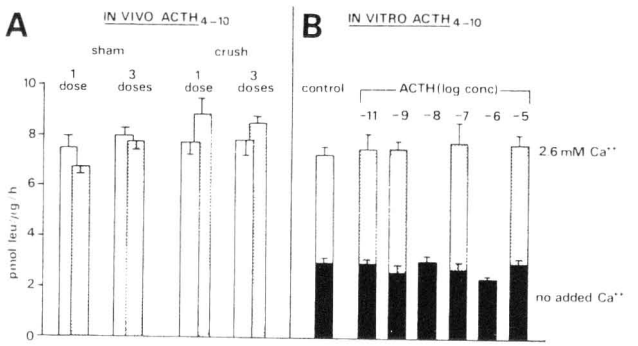

FIG. 1. A: The incorporation of L-[1-^{14}C]-leucine into proteins during a 1 hr incubation *in vitro* was measured in L5 DRG of rats subjected to ipsilateral crush lesions of the sciatic nerve or sham operations and injected with either (dotted box) $ACTH_{4-10}$ (10 μg/rat, SC) or (open box) saline. The effect of a single dose of $ACTH_{4-10}$ was measured in ganglia dissected 90 min after dosing and 24 hr after surgery. The effect of 3 doses of $ACTH_{4-10}$ (administered on days 0, 2 and 4) was measured 4 days after surgery, 90 min after the last dose. The mean and SE of the values from 6 rats for each group are shown. B: L4, L5 and L6 ganglia from normal rats were incubated with L-[1-^{14}C]-leucine for 6 hr *in vitro*. The effect of $ACTH_{4-10}$ at various concentrations (M) in the incubation medium was investigated at different calcium concentrations. The mean and SE from 4 ganglia are shown.

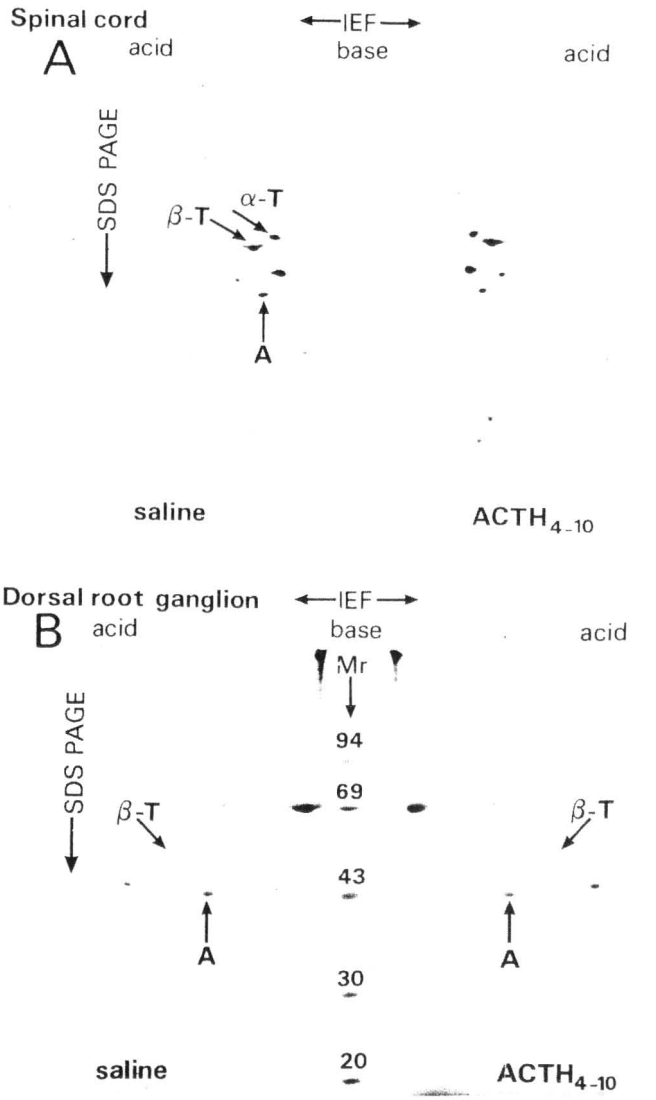

FIG. 2. The protein patterns obtained by 2-dimensional separation of proteins from the spinal cord (A) and L5 DRG (B) of rats that had received a bilateral sciatic nerve crush 4 days previously are shown. The rats were treated with either $ACTH_{4-10}$ or saline as described in the Methods. The positions of α-tubulin (αT), β-tubulin (βT) and actin (A) are indicated. The molecular weights ($10^{-3} \times M_r$) of proteins in a standard mixture of markers are given in B.

final gels were fixed and stained with Coomassie Blue as described by Winter *et al.* [28]. The gels were equilibrated in 10% glycerol, mounted on microscope slides and scanned in the same way as described above for contact prints derived from spinal cord gels.

Measurement of Cytoskeletal Proteins in Sciatic Nerves

The sciatic nerve, together with the tibial branch, was stretched on graph paper and frozen in liquid nitrogen. The nerve was sectioned in 3 mm portions from the proximal boundary of the crush lesion. Each piece was homogenised in 30 μl buffer (as described for the spinal cord, except that sucrose was omitted from the buffer and 15 passes of the homogeniser were used). Samples containing 25 μg protein were mixed with an SDS solution and heated at 60°C for 10 min. Electrophoresis was carried out as described by Zwiers *et al.* [30]. The gels were fixed, stained with Fast Green and photographed under standard conditions. The resultant black and white photographic prints were scanned with a Zeiss M4-QII spectrophotometer at 550 nm.

Protein Assays and Standardisation of Gel Analyses

Total protein concentrations were measured as described by Lowry *et al.* [21], using bovine serum albumin as a standard. The protein content of DRG extracts was measured after TCA precipitation to remove interfering substances in the extraction medium. The positions of actin and α- and β-tubulin in gels were established by comigration with pure standard proteins. Standard curves of stock extracts of the relevant tissues were run and scanned parallel with samples to standardise the assay. The integrated density/area value of each spot in each sample was normalised from the standard curve of the equivalent spot from the stock extract and corrected for the protein content of the sample. The values reported for samples were all within the linear region of the standard curves. Alternative methods of calculating the results (e.g., by normalising to other protein spots within the samples) gave essentially the same results, but the within-group variations were higher (data not shown).

RESULTS

Effect of $ACTH_{4-10}$ on Total Protein Synthesis in DRG

Incorporation of leucine into total proteins in DRG L4, L5 and L6 from sham-operated animals was unaltered by either 1 or 3 doses of $ACTH_{4-10}$ (Fig. 1A shows the results for L5). In rats recovering from a crush lesion of the sciatic nerve, there was a tendency towards slightly higher values in the ACTH-treated group (Fig. 1A), but this was not statistically

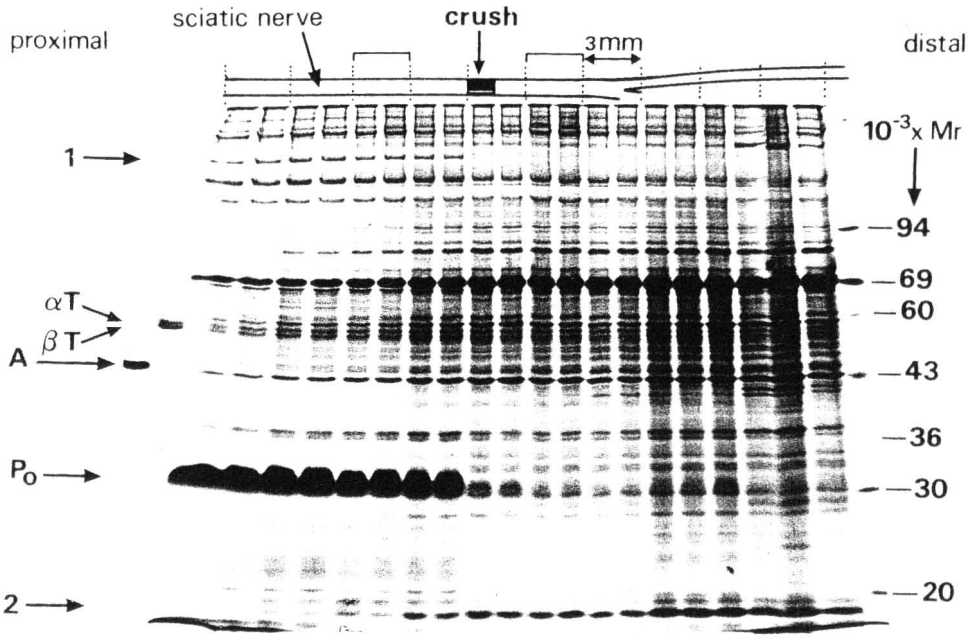

FIG. 3. The proteins present in consecutive 3 mm segments of sciatic nerve are shown in relation to the position of the crush lesion. The extract from each segment was run in duplicate. The gel shown is from a saline-treated rat 8 days after crush lesion of the sciatic nerve. The positions of α-tubulin (αT), β-tubulin (βT) and actin (A) and of 3 proteins (1, Po and 2) that change in response to crush lesion are indicated.

significant. This tendency was probably related to the lower average size (also not statistically significant) of the ganglia from treated rats (see the Method section). No divergence from the control inverse correlation between size and incorporation rate was seen with ACTH treatment.

The presence in the incubation medium of $ACTH_{4-10}$, at concentrations ranging from 10^{-11} to 10^{-5} M, did not alter the rate of protein synthesis (Fig. 1B). The peptide also had no effect when the incubation was carried out in the absence of added calcium, conditions which have been reported to be favourable to demonstration of ACTH effects on protein synthesis in the pineal [25].

Effect of $ACTH_{4-10}$ Treatment on the Amounts of Actin and Tubulin in the Spinal Cord and DRG

Figure 2 shows typical examples of the protein profiles obtained from the spinal cord (A) and DRG (B) of rats that had received a bilateral crush lesion of the sciatic nerve 4 days previously. No consistent differences between the protein patterns from saline- and ACTH-treated rats were seen.

The amounts of α- and β-tubulin and actin, estimated by scanning of the 2-dimensional gels from spinal cords, also showed no effect of $ACTH_{4-10}$ treatment (Table 1). The scanned values for the tubulins in the stock extract of DRG did not increase with increasing extract loaded. The amounts of tubulins in DRG appeared to be very low, compared to that in the spinal cord and peripheral nerves. Values for the tubulin content of this tissue could therefore not be estimated. Actin (together with the majority of proteins in the extract) behaved predictably in the system and the values shown in Table 1 indicate that $ACTH_{4-10}$ treatment does not alter the DRG content of this protein.

TABLE 1

RELATIVE AMOUNTS OF ACTIN, α- AND β-TUBULIN IN SPINAL CORD AND DRG. EFFECT OF $ACTH_{4-10}$ TREATMENT IN CRUSH-LESIONED ANIMALS

	α-tubulin	β-tubulin	Actin
Spinal cord			
saline (6 rats)	28.2 ± 3.8	21.1 ± 2.4	29.7 ± 5.1
$ACTH_{4-10}$ (6 rats)	26.9 ± 4.0	22.7 ± 1.9	28.9 ± 6.1
DRG			
saline (5 rats)	n.d.	n.d.	31.7 ± 12.0
$ACTH_{4-10}$ (5 rats)	n.d.	n.d.	32.0 ± 8.4

n.d.: not determined. The values given are the mean ± SE.

Effect of Nerve Crush and $ACTH_{4-10}$ Treatment of Proteins in the Sciatic Nerve

Proteins present in consecutive segments of the sciatic nerve 8 days after a crush lesion are shown in Fig. 3. The profile in undamaged nerves was indistinguishable from that shown in proximal segments of crushed nerves shown in Fig. 3. The changes in the protein profile observed in degenerating portions of the nerve are similar to those described by others [24,26]. Notably, whereas protein bands at the position of α- and β-tubulin (Fig. 3, αT and βT) and actin (Fig. 3A) were not significantly altered, marked increases in some proteins, e.g., the low molecular weight protein band 2 in Fig. 3 and several bands in the region surrounding α- and β-tubulin, and decreases in others, e.g., band Po (the peripheral nerve myelin protein) and band 1 (probably a high molecular weight neurofilament protein) were observed.

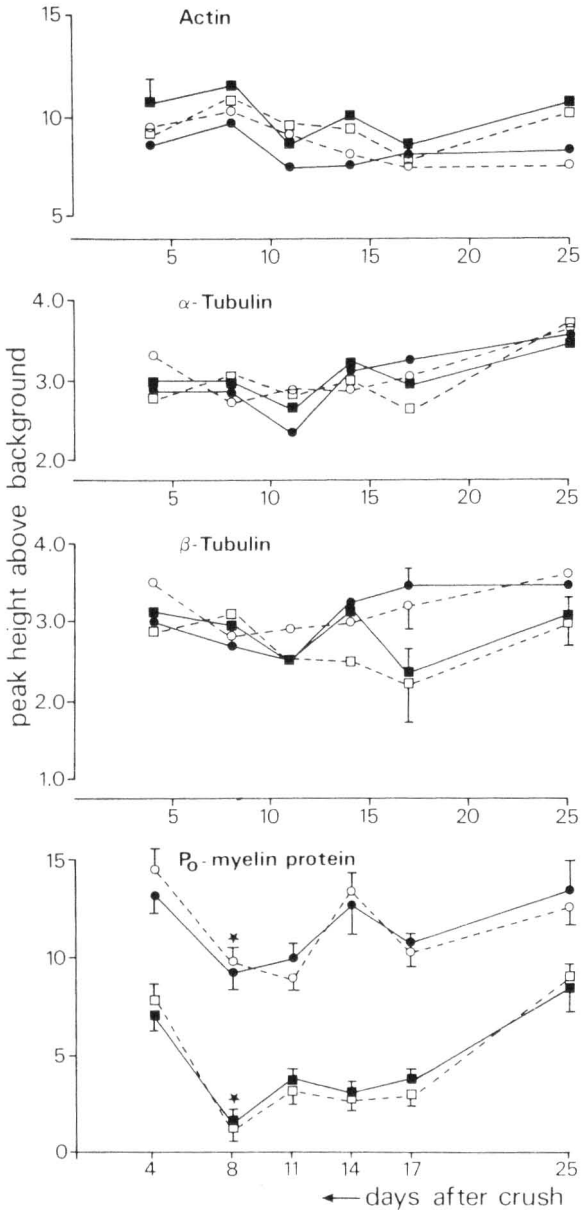

FIG. 4. Peak heights of densitometric scans of peaks corresponding to α-tubulin, β-tubulin, actin and Po in extracts of 3 mm long segments of sciatic nerve 3–6 mm proximal (○,●) and 3–6 mm distal (□,■) to the crush lesion at various times after lesion. The tracks scanned correspond to those marked with brackets in Fig. 3. The values of rats treated with saline (○,□) or $ACTH_{4-10}$ (●,■) are given as means obtained from 6 rats. Bars indicating SE are only given where some divergence between the group means is observed. A significant decrease (Student's *t*-test) in Po between days 4 and 8 (indicated by ★) was observed in the proximal ($0.05<p<0.1$) and distal ($p<0.05$) segments.

The protein patterns of sciatic nerves from $ACTH_{4-10}$-treated rats were indistinguishable from those from saline-treated controls. Densitometric scanning of bands corresponding to α- and β-tubulin, actin and Po (Fig. 4) did not reveal any differences between ACTH- and saline-treated animals at any time up to 25 days after crush lesion. Changes characteristic of degeneration were also seen in the proximal segment of nerve closest to the crush lesion (Figs. 3 and 4). The magnitude of these changes was not altered by ACTH treatment (exemplified by Po in Fig. 4) and in both ACTH- and saline-treated groups, segments more proximal than 6 mm from the crush lesion did not display these changes.

DISCUSSION

The dosing regimens employed in these experiments are identical to those with which we routinely observe the facilitatory effects of $ACTH_{4-10}$ on nerve repair as judged by functional and histological parameters. The earliest measured histological change resulting from ACTH treatment is a large increase in the number of regenerating sprouts observed 3 mm distal to the crush site, eight days after lesion [3]. This would indicate that any effects on the relevant nerve cell bodies would have to have taken place several days previously. Furthermore, the timing of changes induced by the crush lesion itself [16] also suggests that the effects of $ACTH_{4-10}$ on biochemical parameters are most likely to be detected 3–4 days after crush lesion. It is unlikely, therefore, that the failure to observe changes in content of individual proteins or in total protein synthesis can be explained by the timing of the experiment. Moreover, exposure of ganglia for up to 6.5 hr *in vitro* did not alter the rate of total protein synthesis.

It has been suggested [6] that the total volume of axoplasm in regenerating fibres is not altered by ACTH treatment; the increase in axon number being balanced by a concomitant decrease in axon diameter. Thus, an overall increase in protein synthesis is not an essential prerequisite for the increased outgrowth stimulated by ACTH. The significance of changes in overall protein synthesis in the regenerative response is uncertain. Whereas small (30%) increases have been reported in some studies [17], in other systems changes were found not significant [18]. In our experimental model, the crush lesion itself produced only small changes (20–30%) in total synthesis rate and this effect was not observed in all experiments (Edwards, unpublished data). More extensive studies are required to determine whether ACTH alters the synthesis of specific proteins, as has been shown to occur in response to the injury itself [17,18].

Bijlsma [1] reported changes in tubulins and actin in the ventral spinal cord as a result of $ACTH_{4-10}$ treatment in rats recovering from sciatic nerve crush. Using essentially the same experimental protocol, with only minor modifications to improve reproducibility, we found no effects of $ACTH_{4-10}$. The difference between our results and those reported by Bijlsma [1] probably can be explained by the small number of animals employed in the latter study and the difficulty of obtaining reproducible quantitative estimations of proteins in 2-dimensional gels. Such problems are illustrated by the anomalous behaviour of tubulins in DRG extracts. We have found that the non-linearity observed with tubulins was dependent on the tissue sample (data not shown). Atypical behaviour of tubulin has been reported by others [9] and indicates that extreme caution must be exercised in interpreting results of 2-dimensional separations of these proteins. Actin, which behaved predictably in the DRG system, was not altered by $ACTH_{4-10}$ treatment of crush-lesioned rats.

Crush-induced changes that were observed in the protein profiles of portions of sciatic nerve distal to, and immediately proximal to the lesion were similar in saline- and $ACTH_{4-10}$-treated rats. This indicates that ACTH does not significantly alter these degenerative changes.

ACTH-like peptides only facilitate functional recovery if they are given during the period immediately following the lesion [15]. This, together with the early increase in the number of outgrowing fibres, suggests that the mode of action of these peptides must lie in the changes occurring soon after the injury. Several modes of action are possible: (1) an acceleration of Wallerian degeneration leading to decreased mechanical obstruction to outgrowth and increased generation of injury-induced trophic factors, (2) a diminished retrograde degeneration, (3) an enhancement of the early cell body responses to injury, (4) changes in axoplasmic flow facilitating exchange of signals and materials for outgrowth, (5) alterations in the environment of the outgrowing sprouts, and (6) amplification of sprouting signals. Although evidence in the literature (see Introduction) suggested that modes 3 and 4 were plausible, the data presented in this paper do not support such actions. Furthermore, ACTH does not appear to alter the progression of Wallerian or retrograde degeneration. Recent evidence has suggested that ACTH and related peptides may mimic the action of locally produced agents that take part in the trophic influence of degenerating nerve tracts on the outgrowth of axons [15], thus act at 5 and 6 by amplifying sprouting signals generated in the environment of outgrowing sprouts.

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