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Regenerating Neurons

Changes in Protein Phosphorylation

L. Austin,* J. G. Watterson, and M. T. W. Hearn

Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia

Contents

Abstract

We have been studying the phosphorylation of proteins of both normal and regenerating superior cervical ganglia of the rat. Here we report the incorporation of radioactive phosphate into proteins of ganglia homogenates incubated with ^{32}P-labeled ATP under various conditions at day 3 after postganglionic axotomy. The proteins were analyzed by two-dimensional electrophoresis followed by autoradiography. Incubation in the presence of Ca^{2+} or Ca^{2+} plus cyclic AMP produced only about 20 spots corresponding to distinctly labeled proteins. This number was reduced to about five under EGTA plus cyclic AMP conditions, whereas the presence of EGTA alone suppressed the phosphorylation reaction almost totally. All these proteins fell within the narrow p*I* range of 4–6, whereby no qualitative differences between regenerating and control cases were observed. However, the growth-associated protein, variously designated GAP-43, B-50, F-1, and pp-46, had enhanced levels of phosphate incorporation in regenerating ganglia compared to controls. Injury also caused consistently higher levels of phosphorylation of proteins running in the position of α- and β-tubulin. Since these three proteins are major constitutents of regenerating axons, these results suggest that the changes in their phosphorylation induced by injury may be involved in the regulation of their transport.

Index Entries: Superior cervical ganglion; axotomy; nerve regeneration; protein phosphorylation; axonal transport; GAP-43; tubulins.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

We have been using the superior cervical ganglion (SCG) and nodose ganglion of the rat sympathetic nervous system to study biochemical aspects of nerve regeneration. Early results showed that the metabolism of the different forms of RNA varies in a complicated way during the first 2–3 wk after axotomy (Austin, 1985). A consistent feature was the peaking of these RNA changes at day 3 after injury, which thus coincides with the onset of the well-known large-scale morphological changes associated with chromatolysis. Later studies reported on parallel changes in the activities of ribonuclease and its inhibitor in the SCG (Bates et al., 1987), since this enzyme system must play a part in RNA turnover. These studies have been extended to investigate changes in protein phosphorylation in the SCG, which contains the neuronal cell bodies. In particular, attention has been paid to reaction conditions in which the phosphorylation is carried out either on ganglia homogenates (Watterson et al., 1989) or on intact ganglia (Watterson et al., 1990). It was found that at day 3 after injury, there was increased phosphate incorporation into tubulin in the presence of either Ca^{2+} or cyclic AMP in homogenates of regenerating ganglia compared to controls. In addition, the acidic growth-associated protein, variously designated GAP-43, B-50, F-1, and pp-46 (Benowitz and Routtenberg, 1987), also showed enhanced levels of phosphorylation in the regenerating nerve in both homogenized and intact systems. Here we present further studies with ganglia homogenates using different stimulating conditions, the results of which support these earlier findings.

Method

γ^{32}P-labeled ATP was purchased from Amersham and cyclic AMP from Boehringer. Forskolin was a gift from J. O'Neil, Department of Pharmacology, Monash University.

Injuries were performed on female Wistar rats by cutting the post-superior cervical ganglion nerve on the right side, with the left side serving as control. Ganglia were removed usually after 3 d, desheathed, homogenized immediately in a glass/glass homogenizer, and incubated by addition to the radioactive ATP buffer at room temperature. The homogenization buffer was 20 µL of 12 m*M* $MgCl_2$, 125 m*M* KCl, and 10 m*M* Tris, pH 7.3. The ATP buffer was 10–20 µL containing 2–5 µL of stock ATP corresponding to 10–50 µCi of ^{32}P, 30–50 m*M* phosphate, pH 7.0, 0.5% Nonidet P-40, and other additives as indicated below. The incubation was stopped usually after 1 min by addition of 20 µL sodium dodecyl sulfate lysis buffer, and the sample then added to 20 vol of ethanol on ice. The precipitate was collected and analyzed by two-dimensional electrophoresis according to O'Farrell followed by autoradiography, as described elsewhere (Watterson et al., 1989).

Results and Discussion

Here we report studies of the influence of axotomy on the phosphorylation of proteins in the cell body using the SCG. The experimental system we have studied most thoroughly is the incubation of homogenized single ganglia in the presence of ^{32}P-labeled ATP and agents that stimulate the phosphorylation reaction. Previous studies using this in vitro method (Watterson et al., 1989) have shown that over 90% of the ATP, whether at 0.01 or 1.0 m*M* initial concentration, is hydrolyzed within the first minute of incubation, but that only about 1% of the phosphate label becomes covalently bound to protein. Although this level of phosphate incorporation is small, there was a detectable influence of axotomy, whereby incorporation into four protein bands of regenerating ganglia was higher than in control ganglia at day 3 following injury, as revealed by SDS gel electrophoresis.

When ganglionic proteins were analyzed by two-dimensional electrophoresis, the maximum number of well-resolved radioactive

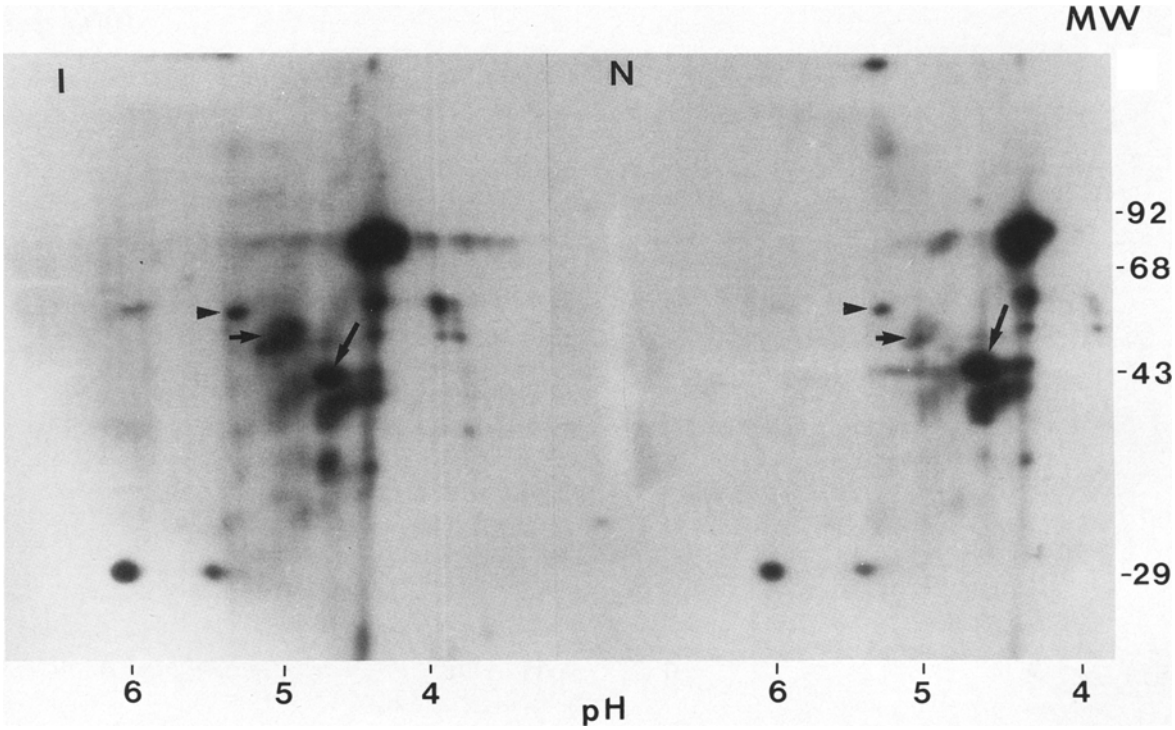

Fig. 1. Autoradiograms of two-dimensional gels of homogenates of single SCG incubated in the presence of γ-^{32} ATP, 0.1 m*M* $CaCl_2$ and 5 m*M* cyclic AMP. I: injured, N: normal. The arrowhead marks α-tubulin, the short arrow marks β-tubulin, and the long arrow marks GAP-43. These were identified by common mobilities.

spots observed was in the range of 20–30. This figure stems from examining 34 autoradiograms of single ganglia incubated under a variety of conditions. By comparison, well over 100 proteins can be readily distinguished in autoradiograms of single ganglia incubated with labeled leucine (Hall et al., 1978). The additional presence of Ca^{2+} ions during the incubation was found to be a more potent condition for stimulating phosphorylation than cyclic AMP. We observed that although Ca^{2+} stimulates the phosphorylation of about 20 proteins seen as clear distinct spots, cyclic AMP produces only about one-quarter as many. In addition, there was no qualitative difference betwen these stimulating conditions, in that those proteins phosphorylated in the presence of cyclic AMP were also labeled in the presence of Ca^{2+}.

Furthermore, no qualitative differences were observed when regenerating and control ganglia were compared, whether phosphorylation was carried out in the presence of Ca^{2+} or cyclic AMP (Watterson et al., 1989). Figure 1 shows autoradiograms of injured and normal cases after incubation of homogenates of single ganglia in the presence of 0.1 m*M* Ca^{2+} plus 5 m*M* cyclic AMP. It illustrates how few spots are finally detected after conducting the phosphorylation reaction under these favorable activating conditions. The pattern of spots resembles very closely that obtained using Ca^{2+} alone.

A dominant feature is the intense, large spot at 85 kDa and p*I* 4.3, which we observed on every autoradiogram independently of the phosphorylation conditions. The two spots migrating at the mol-wt position of 30 kDa at p*I* 5.5 and 6.1 have been observed only under conditions where Ca^{2+} is present and were usually more heavily labeled in the injured case compared to the normal (Watterson et al., 1989). The main difference is the higher level of radioactivity at positions

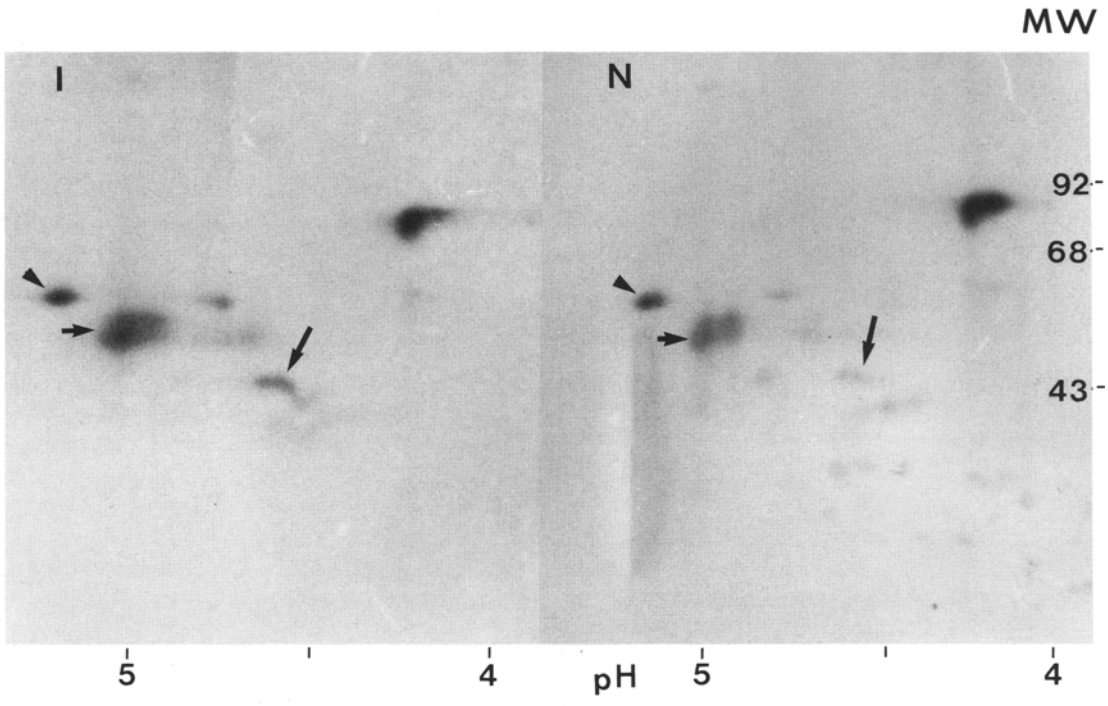

Fig. 2. Autoradiograms of two-dimensional gels of homogenates of single SCG incubated in the presence of γ-^{32}P ATP and 0.25 m*M* forskolin. I: injured. N: normal. The arrowhead marks α-tubulin, the short arrow marks β-tubulin, and the long arrow marks GAP-43.

corresponding to α- and β-tubulin, corroborating our earlier results (Watterson et al., 1989,1990). Perhaps the most surprising feature is the presence of GAP-43. In our studies, it has consistently shown up as an intense spot relative to others and was always visible in autoradiograms from regenerating ganglia, whether incubated intact prior to homogenization, or first homogenized and then incubated in the presence of Ca^{2+} or cyclic AMP. However, the fact that it was usually evident in controls as well speaks to some extent against current dogma concerning this protein. The circumstances of its discovery have indicated its involvement in growth processes, such as axonal extension (Skene and Willard, 1981a; Meiri et al., 1986; Katz et al., 1985; Skene et al., 1986; Kelly et al., 1987), development and neuronal plasticity and potentiation (Aloyo et al., 1983; Nelson and Routtenberg, 1985; Lovinger et al., 1985). Thus, although its presence in the cell bodies of regenerating neurons may be explained, it is not expected to be found in the controls. Our results indicate a more broadly based function than simply growth processes and, since it is now well established to be enriched in axons, suggest a role in transport, but at a much reduced level than in regenerating neurons.

The autoradiograms in Fig. 2 are from incubations carried out in the presence of forskolin, which is a pharmacological agent known to stimulate the adenylate cyclase system (Seamon et al., 1981). Since these autoradiograms are virtually identical to those obtained under cyclic AMP conditions published (Watterson et al., 1989), we may conclude that the enzymatic structures required for cyclic AMP production are active, to some degree at least, in the homogenates. Although qualitative differences between injured and normal were again not evident in this present case, the levels of phosphorylation of both β-tubulin and GAP-43 in the regenerating case were enhanced over their controls. This was a consistent feature

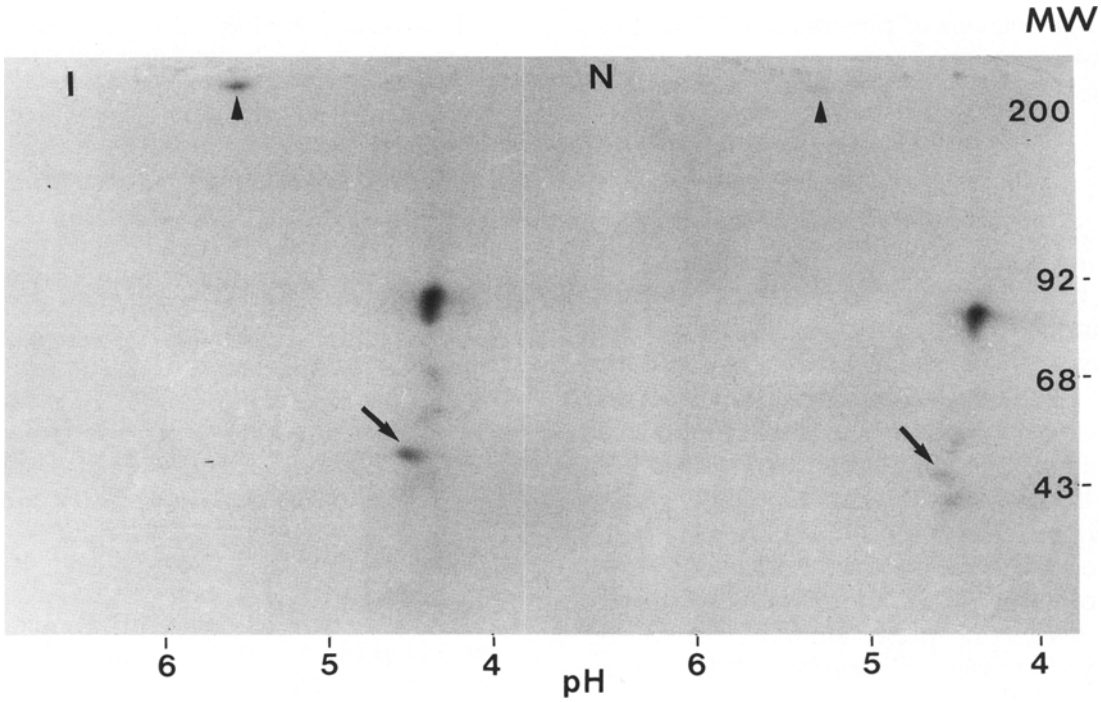

Fig. 3. Autoradiogram of two-dimensional gels of homogenates of single SCG incubated in the presence of γ-^{32}P ATP and 2 m*M* EGTA. I: injured. N: normal. The arrowhead marks MAP-2, and the arrow marks GAP-43

of labeling under cyclic AMP conditions (five experiments). Another reproducible result obtained under this condition was the labeling of α-tubulin, although the phosphorylation of this protein did not show a dependence on injury. In contrast, a spot corresponding to this protein was not always seen in autoradiograms following incubations under high Ca^{2+} conditions, it being visible in 15 out of 24 cases. We can conclude that this variability may result from an uncontrolled state of the cytoskeletal filaments owing to the homogenization, since α-tubulin was always labeled when ganglia were incubated intact prior to being homogenized for electrophoresis (Watterson et al., 1990).

Figure 3 shows the results of incubating in the presence of EGTA. Under this condition, the phosphorylation reaction was almost totally suppressed; however, spots corresponding to the 85-kDa species and GAP-43 are evident. The labeled protein at the top of the gel at p*I* 5.4 is probably MAP-2 as judged from its position, which is now known to be found exclusively in dendrites Gordon-Weeks, 1989). We have observed this protein to become heavily labeled under in vivo conditions, whereby radioactive phosphate is injected into the living animal several hours prior to removal of the ganglia (Watterson et al., 1990). Since it did not enter these 10% gels correctly, its intensity cannot be compared quantitatively to that of other spots. For example, here it appears to be more intense in the regenerating compared to the control ganglion, but in Fig. 1, the situation is reversed. Furthermore, it was not always seen on other autoradiograms. The phosphorylation of these three proteins may have been owing to trace amounts of Ca^{2+}, which were effective even in the presence of 2 m*M* EGTA. However, whatever kinase system is responsible, the presence of these spots indicates how efficiently these proteins become labeled, and suggests that there may be a tight association between the kinase and its substrate protein, which survives the homogenization to an extent that can be detected.

The higher levels of phosphate in GAP-43 in regenerating ganglia compared to controls may be a reflection of its increased expression owing to injury. We have previously presented evidence for its increased abundance based on staining gels for protein (Watterson et al., 1989). It is considered to be primarily an axonal protein, and its association with membranes has been linked to its state of phosphorylation (Gispen et al., 1985; Skene and Willard, 1981b). Our results are in accord with an expected increase in its activities following axotomy, but they further indicate that these changes are under way already in the cell body before transport begins. A similar conclusion can be drawn in the case of tubulin. The phosphorylation of either or both subunits is known to promote its depolymerization (Goto et al., 1985; Yamamoto et al., 1985), and studies on the optic nerve show its phosphorylation to be enhanced by injury (Larrivee and Grafstein, 1987). Taken together with these findings, our results indicate that axotomy causes an increase in the ability of microtubules to occur, not only in the axon, but in the cell body as well.

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