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Time-Dependent Effects of Insulin on Schwann Cell Proliferation in the In Vitro Regenerating Adult Frog Sciatic Nerve

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The present study showed that insulin (0.01 μg/ml, ≈ 2 nM) inhibited [^{3}H]-thymidine incorporation in support cells, most likely Schwann cells, of the cultured frog sciatic nerve. A 25–35% inhibition took place in regenerating nerve preparations as well as in preparations devoid of neuronal protein synthesis, i.e., in isolated 5 mm nerve segments and in gangliectomized nerves, suggesting that the effect was direct and not mediated via the neuronal cells. The inhibition by insulin was time-dependent in that an effect was seen after 4 days but not at shorter or at longer periods of culturing. In separate experiments biotinylated insulin was shown to be taken up by Schwann cells in the regenerating nerve. Addition of serum increased the [^{3}H]-thymidine incorporation severalfold and abolished the inhibitory action of insulin. Our results suggest that insulin, at a certain stage of the regeneration programme, exerts a direct, inhibitory effect on the proliferation of the Schwann cells in the cultured frog sciatic nerve.

Key words: inhibition, nerve regeneration, support cells

INTRODUCTION

A local peripheral injury initiates a series of events in the injured nerve cells and in their surrounding support cells. Via unknown mechanisms the support cells in the injury region are triggered to proliferate. It seems clear that the cell division, consisting mainly of Schwann cells (Pellegrino et al., 1986), is of importance for the outcome of the nerve regeneration. Schwann cell proliferation has been shown to be a prerequisite for proper de- and remyelination of the injured axons (Hall and Gregson, 1977; Love, 1983; Pellegrino et al., 1986; Pellegrino and Spencer, 1985). Moreover, the importance of Schwann cells as a source of substratum and trophic support for the regrowing axons seems to be related to their division, since axonal growth is retarded in situations with reduced proliferation (Pellegrino and Spencer, 1985). However, the influence of proliferation on axonal regrowth could be restricted to the time immediately after the injury. Edbladh et al. (1990) used a blocker of cell division, cytosine-β-D-arabinoside (ara-C) to study the effects of proliferation on axonal outgrowth in the in vitro regenerating frog sciatic nerve. Ara-C at a proliferation blocking concentration inhibited outgrowth when applied during the first 5 days of an 8 day incubation period. In contrast, it was without effect when applied during the last 3 days (Edbladh et al., 1990). In this preparation Schwann cell proliferation seems to influence the initiation of the axonal regrowth rather than its continuation.

The importance of Schwann cell division in the regeneration process stresses the need for clarification of its control mechanisms, particularly those operating during the early stages of regeneration. It is known that certain membrane-bound (Salzer et al., 1980a,b), as well as undefined soluble factors from nerve conditioned media (Le Beau et al., 1988), take part in this regulation, but it seems likely that also other factors are involved. Insulin is a factor of interest in studies of various aspects of nerve regeneration (Ekström et al., 1989). The generality of insulin as a stimulator of metabolism, growth and proliferation (King and Kahn, 1984) also applies to cells of neuronal origin (e.g., Aizenman et al., 1986; Clarke et al., 1984, 1985; Heidenreich and Toledo, 1989; Waldbillig and LeRoith, 1987; Wang et al., 1992) and a role in different nerve functions is likely.

In a previous investigation the effects were tested of low levels of insulin (0.01 μg/ml ≈ 2nM) on protein synthesis and support cell proliferation in the in vitro regenerating frog sciatic nerve (Ekström, 1991). While insulin, in line with other findings (Clarke et al., 1985; Heidenreich and Toledo, 1989), acted as a stimulator of

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neuronal protein synthesis, it unexpectedly attenuated the injury-induced support cell proliferation. To better understand this unusual, inhibitory action of insulin, we have now characterized the effect in more detail with respect to neuronal dependence and time-course of effects. We have also studied if the antimitotic effects of insulin were reflected in changes in support cell protein synthesis and used biotinylated insulin to localize the target cells.

MATERIALS AND METHODS

Frog Nerves

Frogs (*Rana temporaria,* The Frog Farm, Kelly, Ireland) were kept in water tanks at 20°C in an artificial 12 hr light/12 hr dark cycle. Dissection of nerves were as described in detail previously (Edbladh et al., 1990). Briefly, the sciatic nerves were exposed, subjected to a crush lesion and then immediately dissected out with attached 8th and 9th dorsal root ganglia under sterile conditions. The preparations were maintained at 20°C in plastic culture dishes in serum free, HEPES-buffered RPMI 1640 medium, which was changed every second day. For experiments with isolated segments, each uncrushed, outdissected nerve was cut with a scalpel blade to five 5 mm segments, which were cultured as above.

In serum experiments 8.5% fetal calf serum (Gibco) was included in the culture medium.

Insulin

Insulin, porcine zinc-free, was obtained from Calbiochem. Porcine insulin has frequently been used in studies of the endocrinology and developmental biology of the frog (Sadler and Maller, 1987; Scapin and Incerpi, 1992; Schlaghecke and Blüm, 1981). Judging from such reports, there are no reasons to believe that porcine insulin does not react with or has a particularly low affinity for frog insulin receptors.

Thymidine Incorporation

The effects of insulin on the injury-induced proliferation of support cells of the cultured nerves were tested by studying DNA synthesis. After culturing in medium with or without insulin for 4 days, or as indicated, the nerve preparations were incubated in frog Ringer's solution, containing 50 $\mu Ci \times ml^{-1}$ [^{3}H]-thymidine (Amersham), with or without insulin for 4 hr. The nerve preparations were then rinsed with frog Ringer's and 16 mm segments stretching from 8 mm proximal to 8 mm distal to the crush were taken for further analysis. Similarly, the isolated 5 mm nerve segments were incubated with [^{3}H]-thymidine, rinsed and analysed. All nerve segments were then incubated overnight in 10% trichloroacetic acid (TCA) in the cold to extract unincorporated materials. Subsequently, the nerve segments were rinsed in TCA and treated with a tissue solubilizer (Soluene, Packard) prior to assessment of TCA-insoluble materials by liquid scintillation counting. Thymidine incorporation was given as TCA-insoluble cpm $\times$ mm^{-1} nerve.

Leucine Incorporation

To assess the effects of insulin on protein synthesis the incorporation of [^{3}H]-leucine (Amersham) into TCA-insoluble materials was studied. After culturing for 4 days, or as indicated, the nerves were incubated in frog Ringer's solution, with or without insulin, for 4 hr with the addition of 50 $\mu Ci \times ml^{-1}$ [^{3}H]-leucine. The nerve preparations were then rinsed with frog Ringer's and 16 mm segments, stretching from 8 mm proximal to 8 mm distal to the crush, were incubated overnight in 10% TCA in the cold. After a rinse in TCA each nerve segment was subjected to hydrolysis in 1 M NaOH overnight in the cold. Aliquots of this solution were then taken for liquid scintillation.

Uptake of Insulin

Uptake of insulin into cells of the frog sciatic nerve was studied at the light microscopic level with biotinylated bovine insulin purchased from Sigma.

Nerves that had been cultured for 4 days in vitro in the absence of insulin were incubated in biotin-insulin containing medium (100 μg/ml) overnight at 20°C. After rinsing in 0.1 M phosphate buffered saline, pH 7.4 (PBS), the nerves were fixated in 4% paraformaldehyde in PBS overnight, cryoprotected in PBS with 30% sucrose for 24 hr and cut in 9 μm sections in a cryotome. Sections were then incubated for 60 min with streptavidin-biotinylated horse radish peroxidase complex (Amersham), 1:50 in PBS. The localization of the biotin-insulin was visualized by diaminobenzidine and H_2O_2 according to the manufacturer's protocol (Sigma). Following this, sections were rinsed, dehydrated in a graded series of ethanol and finally in xylen, mounted with DPX (BDH) and viewed in a Leica Laborlux microscope with an attached Sony videocamera and videoprinter.

To assess the specificity of the insulin uptake the paired, contralateral nerves were incubated with 100 μg/ml biotin-insulin plus an excess (2,000 μg/ml) unlabelled insulin.

As an alternative, porcine insulin (1 mg $\times$ ml^{-1} in 0.1 M Na HCO_3, pH 8.0) was biotinylated with N-hydroxysuccinimidobiotin (Sigma), final concentration 750 μM, in an overnight reaction at 20°C. After quenching by addition of 0.1 M Tris, insulin was separated from the labelling reagents by means of gel filtration in a PD-10 column (Pharmacia), equilibrated with frog Ringer's solution. The gel filtrated biotin-insulin was stored at

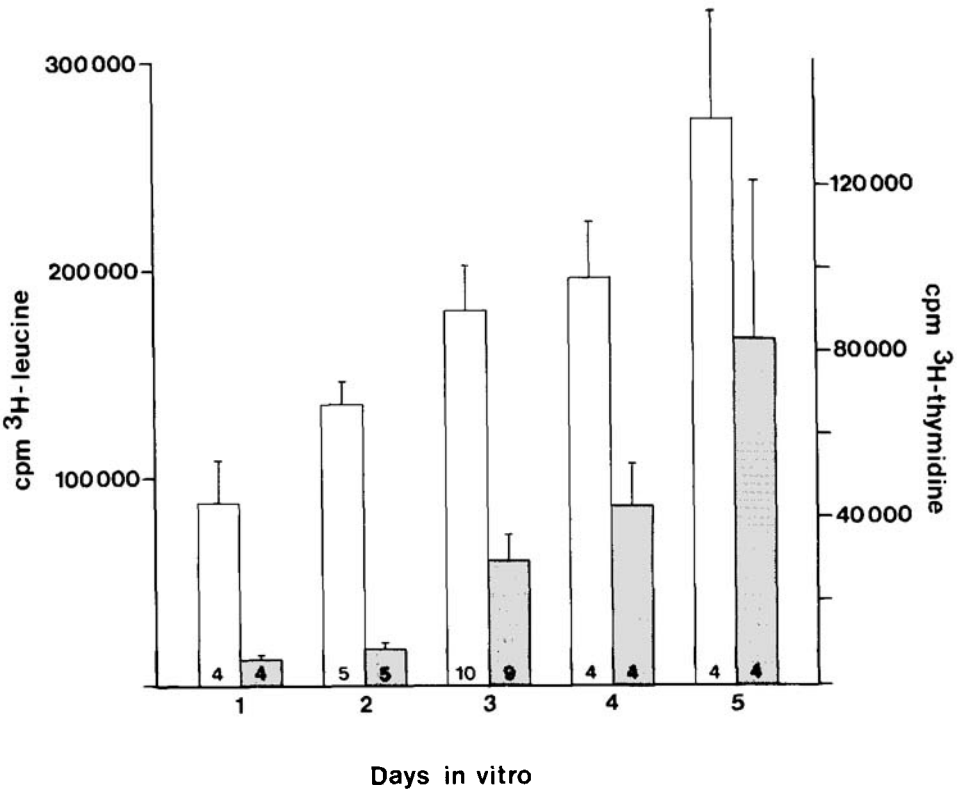

Fig. 1. Incorporation of [^{3}H]-leucine and [^{3}H]-thymidine in regenerating frog sciatic nerves during the final 4 hr following different times in vitro. Results are expressed as TCA insoluble radioactivity in 16 mm segments containing the nerve crush (mean ± SEM). The number of nerves used for each observation is indicated at the bottom of the bars. Unfilled bars represent [^{3}H]-leucine incorporation; left axis. Shaded bars represent [^{3}H]-thymidine incorporation; right axis.

−18°C at a concentration of 0.7 mg × ml^{-1} frog Ringer's.

Nerves were incubated in the biotin-insulin containing frog Ringer's with 1% bovine serum albumin overnight at 20°C. After rinsing in frog Ringer's, the nerves were fixated in paraformaldehyde, rinsed in PBS and cut in 7 μm sections in a cryotome. Sections were then incubated for 60 min with FITC-avidin, 1:50 in PBS, rinsed, mounted and viewed in a Leica Laborlux fluorescence microscope with an attached camera.

RESULTS

Kinetics of Leucine and Thymidine Incorporation in the Frog Nerve

First, the kinetics of [^{3}H]-leucine and [^{3}H]-thymidine incorporation in 16 mm nerve segments after various times in culture were established. Figure 1 shows the results from experiments, in which one nerve from each paired preparation was incubated in [^{3}H]-leucine and the other in [^{3}H]-thymidine for a 4 hr period at the end of the culturing. The longer the nerves had been cultured, the higher was protein- and DNA-synthesis. The proportionally biggest difference between two succeeding days of culturing was between day 1 and 2 for protein-synthesis and day 2 and 3 for DNA-synthesis, with a 53% and 234% increase, respectively.

Effects of Insulin on Thymidine Incorporation

The effects of insulin (0.01 μg × ml^{-1}) on [^{3}H]-thymidine incorporation in nerve preparations cultured for various time periods are shown in Figure 2. There was a significant inhibition (64% of control) of thymidine incorporation in preparations exposed to insulin for 4 days, whereas shorter or longer exposure times lacked effects.

Thymidine incorporation after insulin treatment of different preparations of nerves and isolated segments after 4 days of culturing is presented in Table I. Insulin reduced the DNA synthesis of support cells of the sciatic nerve regardless if the latter was cultured as in vitro regenerating nerves with attached ganglia, as nerves with excised ganglia or as isolated 5 mm segments. The effect of gangliectomy per se was studied in separate experiments, showing that excision of the ganglia did not affect the DNA synthesis of the support cells over a 4 day in

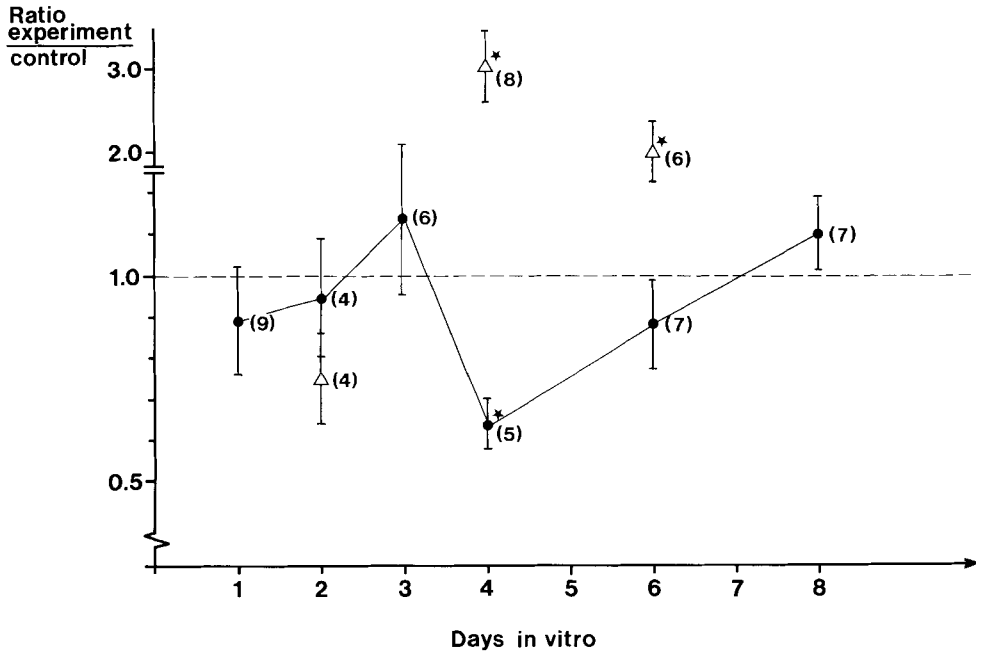

Fig. 2. The effect of various culturing times on the incorporation of [^{3}H]-thymidine in the support cells of the in vitro regenerating frog sciatic nerves in the presence of 0.01 μg/ml insulin (●) or 8.5% serum (△). Paired nerves were cultured with or without the addition of insulin or serum and labelled during the final 4 hr of the in vitro periods. Thymidine incorporation was measured in 16 mm segments containing the nerve crush. Results are expressed as ratios of the values of TCA insoluble radioactivity in paired preparations (mean ± SEM). The number of preparations used for each time-point is indicated by a figure. Asterisks indicate a value statistically different (Student's one-group t-test, $P<0.05$) from the ratio of 1. Note the difference in scale for ratio values > 1.2.

vitro period (ratio of thymidine incorporation in nerves without ganglia / nerves with ganglia: 1.08 ± 0.22, mean ± SEM, n = 5, not significant, n.s.). Addition of serum strongly stimulated the DNA synthesis and abolished the inhibiting effect of insulin on the regenerating nerves. Also the proliferative response to serum varied over time (Fig. 2), with the largest effect after 4 days of culturing.

Table II gives the results from thymidine incorporation experiments under various other conditions. Firstly, it is shown that also a lower (0.005 μg × ml^{-1}, ≈1 nM) concentration of insulin inhibited the proliferation of support cells. Secondly, the table shows that the inhibitory effect of insulin at 4 days was not dependent on continuous insulin exposure, nor was it the result of an interference with the mechanisms that induce the proliferative response. DNA-synthesis in nerves, that had been regenerating for 4 days in vitro and then cultured for yet another day with or without insulin was still significantly inhibited by insulin. The reverse experiment, i.e., freshly dissected nerves, cultured with or without insulin for 1 day prior to a 4 day in vitro period in the absence of insulin, did not reveal any effect of insulin.

Effects of Insulin on Leucine Incorporation

To study whether the inhibited DNA synthesis by insulin reflected a general inhibitory effect on metabolism, the amount of [^{3}H]-leucine incorporation was measured. Regardless if the radioactivity was related to mm nerve or mg total protein, the presence of 0.01 μg × ml^{-1} insulin in 4 days culturing experiments did not affect the incorporation of [^{3}H]-leucine in 16 mm nerve segments (ratios of insulin to control: 0.96 ± 0.09 cpm × mm^{-1}; 0.99 ± 0.10 cpm × mg^{-1}, n = 6, n.s.). Likewise, incorporation of [^{3}H]-leucine was not changed by insulin in nerves cultured for 2 days (ratio: 1.04 ± 0.11, n = 6, n.s., measured as cpm × mm^{-1}). This suggests that insulin treatment did not result in stimulation of protein synthesis early in the regeneration process, when synthesis showed the biggest increase (Fig. 1).

Uptake of Insulin

Figure 3 shows that biotin-insulin after an overnight incubation was taken up into cells that had the characteristics of Schwann cells of the myelinated fibers. Figure 3A,B is from experiments with biotinylated por-

TABLE I. Incorporation of [^{3}H]-Thymidine in TCA-Insoluble Fraction of Frog Sciatic Nerves After 4 Days of Culturing[†]

		In vitro regenerating nerves[a]			Nerves without ganglia[b]	Isolated nerve segments[c]
Additions to medium:						
Experiment (E)	=	Insulin	Serum	Serum + Insulin	Insulin	Insulin
Control (C)	=	None	None	Serum	None	None
cpm [^{3}H]-thymidine	E =	4,318 ± 852 (5)	10,066 ± 1,580(8)	13,181 ± 2,391(18)	2,973 ± 1,166(4)	1,733 ± 380 (7)
mm^{-1} nerve	C =	6,524 ± 749	3,866 ± 898	12,480 ± 1,910	4,525 ± 1,908	2,219 ± 360
Ratios E/C		0.64 ± 0.06	3.05 ± 0.42	1.18 ± 0.15	0.71 ± 0.06	0.75 ± 0.07
Different from 1.0[d]		*	*	n.s.	*	*

[†]Values are given as mean ± SEM, numbers of paired observations are given within brackets.
Additions to the culture medium: insulin = 0.01 μg × ml^{-1} porcine insulin, serum = 8.5% fetal calf serum. See text for further details.
[a]Each observation is based on the cpm-value of a 16 mm nerve segment stretching from 8 mm proximal to the crush to 8 mm distal to the crush, taken from complete but crushed sciatic sensory nerves incubated for 4 days under the indicated conditions.
[b]As for [a] except that the crushed nerves were incubated without their ganglia.
[c]Each observation is based on the average cpm-value of five 5 mm nerve segments that had been incubated for 4 days under the indicated conditions.
[d]Statistical analyses of the ratios were performed with Student's one-group t-test. Population mean was set to 1.0 (an E/C ratio of 1.0 would thus indicate no difference).
*Indicates a significant difference ($P<0.05$) from 1.0, n.s. = not significant.

cine insulin, which was detected by fluorescent avidin. Label was seen only in the vicinity of the crush and was located to parallel structures, which at places had a reduced interdistance and that sometimes connected with each other. This is consistent with the configuration of Schwann cell cytoplasm in myelinated fibers that go in and out of the plane of section. Figure 3C,D is from experiments with biotinylated bovine insulin, detected with peroxidase conjugated avidin. As with the porcine insulin label was seen in Schwann cells, whereas sheath cells and endothelial cells were not labeled. The presence of an excess of unlabelled insulin prevented the uptake of biotinylated insulin (Fig. 3D), indicating that the uptake was specific, i.e. receptor mediated. Similar results were obtained in parallel experiments where lower concentrations of biotin-insulin and unlabelled insulin were used (2 μg/ml and 200 μg/ml, respectively), although the labelling was considerably weaker (not shown).

DISCUSSION

The present study concerns the effect of insulin on the proliferation of support cells in the cultured frog sciatic nerve. Before discussing the effects of insulin, it seems appropriate to briefly deal with the question of initiation of mitosis, a matter of current debate. The present as well as earlier studies (Edbladh et al., 1990; Edström et al., 1990; Ekström, 1991) show that under culture conditions the frog sciatic nerve displays proliferation of Schwann cells, suggesting that the mitosis signal is to be found within the preparation. Likewise, proliferation of Schwann cells has been demonstrated in cat sciatic nerve explants (Crang and Blakemore, 1986). On the other hand, some authors point out non-resident macrophages as the initiators of Schwann cell mitosis (Baichwal et al., 1988; Beuche and Friede, 1984). Although explanted peripheral nerves may contain a small percentage of macrophages (Beuche and Friede, 1984; Ekström et al., unpublished observations), their role in Schwann cell mitosis is considered to be insignificant (Beuche and Friede, 1984). Therefore, the initiation signal seems to have a different origin. However, the signal is clearly not dependent on neuronal macromolecular synthesis, since in the present study proliferation also occurred in isolated nerve segments and was not affected by gangliectomy. For the same reasons, the initiating factor could not be the membranes of growing axons, as suggested by Salzer et al. (1980a,b). Remaining candidates for the initiation signal include direct mechanical injury (Salzer and Bunge, 1980), soluble compounds produced in the vicinity of the injury (LeBeau et al., 1988), and loss of an axonal component (Pellegrino et al., 1986). For the cultured, regenerating frog nerve, none of these explanations can be excluded.

In accordance with previous results (Ekström, 1991), nanomolar concentrations of insulin in the present study inhibited the proliferation of support cells in the cultured frog sciatic nerve. The proliferation in the frog nerve is largely accounted for by Schwann cells (Edström et al., 1990), which here were shown to take up biotin-insulin, suggesting that the inhibition involves these cells. In theory, however, insulin could have inhibited Schwann cell proliferation via other cells, for instance the sciatic sensory neurons, which are activated by insulin treatment (Ekström, 1991). This possibility can now be dismissed, because inhibition occurred regardless if regenerating nerves, nerves without ganglia, or isolated segments were studied.

TABLE II. Incorporation of [^{3}H]-Thymidine in TCA-Insoluble Fraction of Frog Sciatic Nerves After Treatment as Indicated†

Experiment (E)	=	4 days in vitro with 0.005 μg × ml^{-1} insulin	4 days in vitro + 1 day in vitro with 0.01 μg × ml^{-1} insulin	1 day in vitro with 0.01 μg × ml^{-1} insulin + 4 days in vitro
Control (C)	=	4 days in vitro	5 days in vitro	5 days in vitro
cpm [^{3}H]-thymidine × mm^{-1} nerve	E =	3,536 ± 865 (12)	3,444 ± 678 (5)	5,047 ± 853 (6)
	C =	5,073 ± 993	4,376 ± 938	5,509 ± 453
Ratios E/C[a]		0.67 ± 0.08 *	0.82 ± 0.06 *	0.93 ± 0.15 n.s.

†Each observation is based on the TCA-insoluble radioactivity of a 16 mm nerve segment stretching from 8 mm proximal to the crush to 8 mm distal to the crush. All culturing was performed in serum-free medium. Values are given as mean ± SEM, numbers of paired observations are given within parentheses.

[a]Statistical analyses of the ratios were performed with Student's one-group t-test as for Table 1. *Indicates a significant difference ($P < 0.05$) from 1.0, n.s. = not significant.

The experiments revealed that for the cells in the crush region of the cultured nerves an increase in protein synthesis precedes the increase in DNA synthesis by approximately 1 day. The regeneration programme leading to multiplication of cells therefore seems to be activated before there are actual signs of mitosis. Insulin failed to affect protein synthesis in cultured nerves both after 2 days, when the culture-related increase in protein synthesis is biggest, and 4 days, when the insulin-inhibition of DNA synthesis is biggest. This means that insulin does not interfere with either the general metabolism of the cells or the activation of the regeneration process, at least as expressed in an increased protein synthesis. Instead the effects of insulin is focused on the DNA synthesis seen after culturing for 4–5 days, with no responses at shorter or longer culturing periods. Stimulation by serum showed a similar time-dependence, with the greatest effect after 4 days of culturing. The proliferation of Schwann cells could therefore be specifically controlled, for instance by insulin, at certain stages after an injury, which is a novel finding.

A direct, anti-proliferative action of insulin may appear controversial in light of its well documented stimulation of growth and metabolism. Although an anti-proliferative effect on transformed cells by insulin has been known for several years (Kahn et al., 1980), the behaviour of such cells may not readily be extrapolated to normal, undissociated cells. However, very recently an inhibitory effect by insulin on the early development of the kidney was described by Chailler and Brière (1991). The proliferation of kidney cells was reduced by insulin, but only during a limited period of the development. Likewise, Tesoriere et al. (1992) reported that insulin in the nanomolar range reduced thymidine incorporation in explants of chick embryo retina during a specific stage of their embryonic development. The latter effects were proved not to be due to increased degeneration. The findings indicate that the Schwann cells of the frog nerve are not unique with regard to their inhibitory response to insulin.

Despite the broad range of insulin concentrations tested in the present and a previous study (0.005 μg × ml^{-1}–10 μg × ml^{-1} ≈ 1 nM–2 μM, Ekström, 1991), the inhibitory effect never amounted to more than 36%. Likewise, the inhibitory effect of insulin on retinal cell proliferation could not be increased to more than 65% (Tesoriere et al., 1992). The functional significance of the partial inhibition by insulin seen in these cases is not clear, but could represent the suppression of certain cell populations at a given developmental or post-injury stage. If so, the influence of other cells, that have properties of particular importance during these periods, would be enhanced. As another possibility, insulin could be a differentiating factor for some cells, whose increased grade of differentiation then is reflected in a decreased DNA-synthesis.

Addition of serum to the incubation medium prevented the inhibitory effect of insulin in the regenerating nerves (Table I), the reason for which is not known. Serum is largely uncharacterized and contains many factors with unknown functions, that could have acted to normalize the effects of insulin. Alternatively, the inhibitory effect of insulin may be outweighed by the stimulatory effect of serum. This property of serum may have excluded inhibitory effects of insulin from discovery in such cell and tissue culture systems, where serum is required for survival and maintenance. It is therefore interesting to note, that inhibition by insulin in retinal explants as well as in the developing kidney was seen under serum-free conditions (Tesoriere et al., 1992; Chailler and Brière, 1991).

In conclusion, we report of an inhibitory effect by insulin on the injury-induced proliferation of Schwann cells in the frog sciatic nerve. Inhibition occurred at low concentrations of insulin and was time-dependent. In this respect it resembled the inhibition seen in developing cells of some other tissues. The results are compatible with a regulatory role for insulin at certain stages of the cellular growth taking place in the injured peripheral nerve.

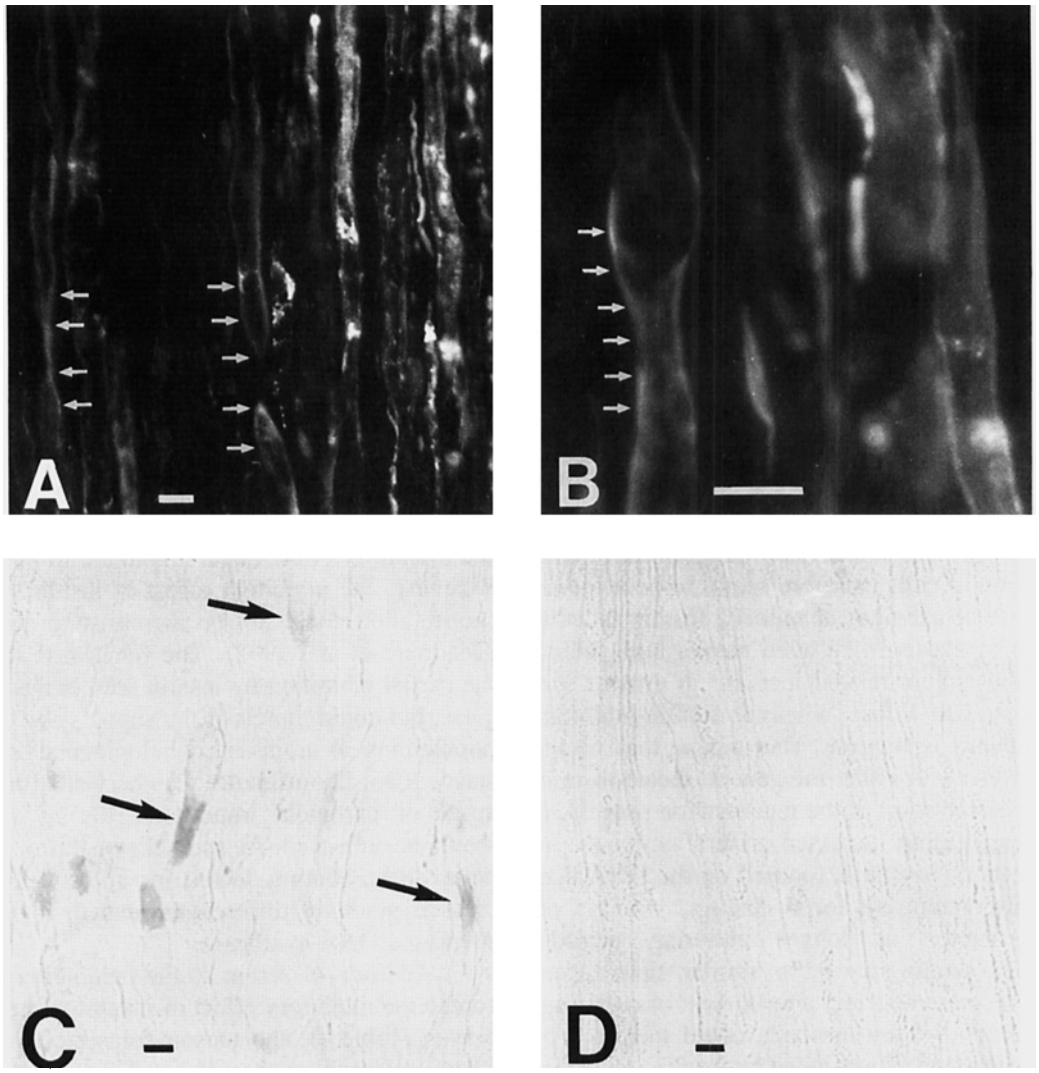

Fig. 3. Longitudinal cryotome sections of crush-injured frog sciatic nerves incubated in vitro for 4 days before an overnight incubation with biotin-insulin. Uptake of biotinylated substances was visualized by treatment with fluorescent avidin (**A,B**) or a streptavidin-horse radish peroxidase complex and diaminobenzidine (**C,D**). **A:** Immediately distal to the crush region of a sciatic nerve incubated with biotin-insulin. Label is present in Schwann cell cytoplasm (arrows), surrounding myelinated fibers. **B:** As A but greater magnification. **C:** Uptake of biotin-insulin in Schwann cell cytoplasm (arrows) immediately distal to the crush. **D:** A section corresponding to that in C of a nerve incubated in biotin-insulin plus unlabelled insulin. No reaction products can be seen. Scale bars are 10 μm.

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