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CYCLIC AMP AND CALCIUM AS POTENTIAL MEDIATORS OF STIMULATION OF CULTURED SCHWANN CELL PROLIFERATION BY AXOLEMMA-ENRICHED AND MYELIN-ENRICHED MEMBRANE FRACTIONS

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The roles of cyclic AMP and calcium in the transduction of the mitogenic effects of central nervous system axolemma and myelin-enriched fractions on cultured Schwann cells were examined. Cyclic AMP levels were not elevated in axolemma or myelin-stimulated Schwann cells, but were increased when stimulated with cholera toxin, an adenyl cyclase activater. The mitogenicity of axolemma and myelin was markedly reduced by 2.5 mM citrate, a calcium chelator, and 10 uM trifluoroperazine, an inhibitor of calmodulin. Treatment of Schwann cells with several tumor-promoting phorbol esters caused significant enhancement of the mitogenicity of the axolemma and myelin preparations. These data suggest that the mitogenic effects of axolemma and myelin are not mediated by cyclic AMP, but may be mediated by calcium ions.

Schwann cells are known to proliferate *in vivo* during both Wallerian degeneration (1,2) and neural development (3,4). These phenomena have been studied *in vitro* using neurally-derived substances which have been shown to be mitogenic for cultured Schwann cells, including neurites from dorsal root ganglia (5), a pituitary-derived growth factor (6), axolemma-enriched fractions from both the peripheral and central nervous systems (7,8), and myelin (9).

The role of cAMP[3] in mediating this proliferative response is controversial. Cholera toxin, an adenyl cyclase activater, and cAMP analogs are known to stimulate Schwann cell mitosis (5,6,7,10). When measured in proliferating

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[3] Abbreviations: cAMP, cyclic adenosine 3':5' monophosphate; PDB, phorbol 12,13-dibutyrate; Phr, phorbol (parent alcohol); PMA, 4β-phorbol-12-myristate-13-acetate.

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Schwann cells stimulated with cholera toxin, large increases in cAMP concentrations have been observed (6). No significant elevation of cAMP levels has been observed, however, in Schwann cells stimulated with pituitary extracts (6,11). Salzer and Bunge (5) presented indirect evidence that neurite-stimulated Schwann cells did not utilize cAMP as a second messenger, although a recent report (12) demonstrated minimal increases in intracellular Schwann cell cAMP levels when stimulated with neurites derived from PC-12 tumor cells and dorsal root ganglia.

In this communication, we report for the first time on the effect of stimulation by axolemma-enriched and myelin-enriched membrane fractions on the intracellular cAMP levels in cultured Schwann cells. We also present preliminary data suggesting that calcium may serve as a physiological second messenger during membrane stimulated Schwann cell proliferation.

MATERIALS AND METHODS

Preparation of Schwann Cells. Schwann cells were prepared by modifying the method of Brockes, et. al (13). Briefly, sciatic nerves were removed from 2-3 day rat pups. The nerves were treated with Trypsin (Type IX, Sigma) and collagenase (Type III, Worthington), then were triturated with a pasteur pipet and filtered through 209 u Nitex (Tetko). The cells were collected by centrifugation and resuspended in Dulbecco's Modified Eagle's (GIBCO) with 10% Fetal Calf Serum (Sterile Systems). The cells were plated in 100 mm glass dishes at a density of 3-4.5 X 10^6 cells per dish. Cytosine arabinoside (10^{-5}M) was added after 24 hours to inhibit fibroblast proliferation. The cytosine arabinoside was removed after 72 hours. Following an additional 3 days, remaining fibroblasts were eliminated by complement-mediated lysis with anti-Thy 1.1 (New England Nuclear) and rabbit complement (Cappel Labs). The cells were plated onto 12mm glass coverslips at a density of 3-5 X 10^4 cells per slip. When examined by phase microscopy, the cultures were greater than 99.5% Schwann cells.

Preparation of Axolemma-Enriched and Myelin-Enriched Membrane Fractions. Axolemma-enriched and myelin-enriched enriched fractions were prepared from rostral bovine cervical cord white matter by the method of DeVries, et. al (14) in the presence of 0.02 % azide. Sterile aliquots of the preparation were rinsed free of azide, and frozen (-20° C.) at protein concentrations of 1 mg/ml. Protein concentrations were determined by the method of Bradford (15).

cAMP determinations. cAMP levels were determined by radioimmunoassay by the method of Steiner, et. al (16), with a kit from New England Nuclear. The protocol outlined by Raff, et. al (6) for measuring cAMP in stimulated Schwann cells was utilized.

Light Level Radioautography. The mitogenicity of various preparations was determined by their ability to facilitate [^{3}H] thymidine incorporation into Schwann cells as measured by radioautography. Schwann cell-plated coverslips were contained in 24 well Linbro plates, in 500 uL of Dulbecco's Modified

Eagle's + 10% fetal calf serum. Axolemma and myelin (final concentration = 14 ug protein/ml), and other agents were added 24 hours after plating. Cholera toxin (Miles Laboratories) was used at a final concentration of 7 ug/ml. Trifluoroperazine (a gift from Dr. S. Laychock), Phr, PMA, PDB (gifts from Dr. R. Carchman), and sodium citrate (Sigma) were added at the final concentrations indicated in relevant figures. All solutions were prepared sterilely by filtration.

Twenty-four hours after these additions, 1.5 uCi (62.5uL) of [^{3}H]thymidine (19.3 Ci/mmol, New England Nuclear) was added. The final volume in each well was 712.5 uL. The cells were prepared for radioautography 48 hr after the addition of thymidine by a previously published method (7,9). At least 5 fields were counted per coverslip, for a minimum total of 500 cells. A cell was considered labelled if at least 20 grains were over the nucleus.

Statistics - Student's t-test was utilized for all statistical analyses, with $p < 0.05$ used to define significance. Results are reported as mean percentages ± S.E.M.

RESULTS

To ascertain whether cyclic AMP levels changed during axolemma and myelin stimulation of Schwann cell proliferation, cultures were treated with axolemma, myelin, cholera toxin, or nothing (control) for 3,6 and 9 hours, after which intracellular cAMP levels were determined. Figure 1 summarizes a typical experiment. cAMP levels in axolemma and myelin-treated cells were not significantly different from control values during the experiment; cholera toxin caused a highly significant increase in cAMP levels, confirming previous reports (cf,6).

The possible role of calcium as a second messenger was also examined, inasmuch as calcium and cyclic nucleotide-mediated effects are often similar (17). To determine if calcium is necessary for expression of the mitogenic

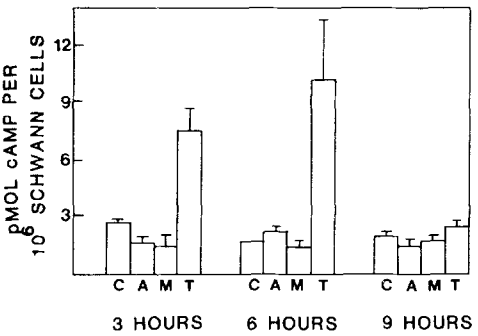

Figure 1. Intracellular cAMP levels in mitogen treated Schwann cells at various times following mitogen addition. C, control (no added mitogen); A, axolemma; M, myelin; T, cholera toxin. Schwann cells were plated at a density of 57000 per well. Results are means of determinations of two separate wells in duplicate (± S.E.M.). Only 3 and 6 hr. cholera toxin values are significantly different from control levels ($p<0.05$).

TABLE I. Effect of Perturbing Calcium Homeostasis on Cultured Schwann Cell Response to Mitogens

Treatment	% [^{3}H] Thymidine Labelled Schwann Cells	
	Axolemma-Treated	Myelin-Treated
Control-no addition	12.5 ± 1.0	10.5 ± 2.6
10 uM trifluoroperazine	1.1 ± 0.3	0.76 ± 0.35
2.5 mM citrate	2.0 ± 0.9	2.5 ± 0.4

Control wells (with all additions but no axolemma or myelin) consistently had $<$0.2% labelled Schwann cells.

signals of axolemma and myelin, extracellular free calcium ion concentration was reduced 80% by chelation with 2.5 mM citrate (25). Citrate treatment did not alter the Schwann cell morphology, but did cause an 84% reduction in the mitogenicity of axolemma, and a 76% reduction in the mitogenic activity of myelin, as measured by their ability to facilitate incorporation of [^{3}H] thymidine into Schwann cells (Table I).

Many cellular calcium mediated events require calcium binding to the protein calmodulin for expression (17,18). The involvment of calmodulin can be inferred by using the drug trifluoroperazine, which inactivates calmodulin by binding to the protein is the presence of calcium (17, 19). The mitogenic signals of axolemma and myelin were reduced 91% and 93%, respectively, in the presence of 10 uM trifluoroperazine.

The effect of phorbol esters on the expression of the mitogenic signals of axolemma and myelin was also examined (Table II). The tumor promoting esters (PMA and PDB, in this report) have numerous cellular effects, including the ability to facilitate an inwardly-directed calcium flux following their binding to the cell surface (20), and to activate calcium-dependent, phospholipid-sensitive protein kinase (21, 22) by replacing diacylglycerol as a cofactor (23). Schwann cells were treated with axolemma or myelin in the presence or absence of 10^{-6} to 10^{-8}M PMA, PDB (tumor-promoting) or Phr (inactive). As shown in Table II, PDB at all tested concentrations significantly increased the mitogenicity of both axolemma and myelin. PMA increased

TABLE II. Effect of Phorbol Esters on Cultured Schwann Cell Response to Mitogens

Experiment	Treatment	% [^{3}H]Thymidine Labelled Schwann Cells	
		Axolemma-Treated	Myelin-Treated
I	Control - no addition	12.5 ± 1.0	10.5 ± 0.9
	Phr 10^{-8} M	13.1 ± 1.5	10.1 ± 1.2
	PMA 10^{-8} M	9.1 ± 1.1**	8.9 ± 1.5
	PDB 10^{-6} M	34.1 ± 0.7*	19.0 ± 3.6*
	PDB 10^{-7} M	29.2 ± 3.6*	20.7 ± 1.7**
	PDB 10^{-8} M	ND	20.1 ± 1.7*
II	Control - no addition	6.9 ± 0.9	5.6 ± 0.7
	Phr 10^{-6} M	6.4 ± 0.9	6.0 ± 1.2
	Phr 10^{-7} M	6.6 ± 0.8	5.0 ± 0.3
	PMA 10^{-6} M	7.1 ± 0.3	12.1 ± 0.8*
	PMA 10^{-7} M	14.8 ± 1.2*	14.8 ± 0.7*
III	Control - no addition	16.8 ± 1.6	12.2 ± 0.7
	Phr 10^{-7} M	17.4 ± 1.2	13.9 ± 1.3
	PMA 10^{-7} M	19.3 ± 1.6	16.1 ± 0.7*
	PDB 10^{-7} M	37.2 ± 2.1*	18.7 ± 1.7*

*, $p < 0.001$; **, $p < 0.05$

the mitogenicity of myelin at 10^{-6} -10^{7}M, but not at 10^{-8}M. The mitogenicity of axolemma was increased by 10^{-7}M PMA in one experiment, but not in another; 10^{-8}M PMA had no effect and 10^{-6}M PMA actually reduced the mitogenicity of axolemma. Phr, the inactive ester, had no effect on the mitogenicity of either axolemma or myelin. Control wells (i.e., Schwann cells treated with or without phorbol esters but not axolemma and myelin) typically had 0-2 labelled cells per 500 (< 0.4 %), except in the case of 10^{-7}-10^{-8}M PDB, in which the labelled cells were up to 1-2% of the total cell count. This slight increase was far too small, however, to account for the enhancement seen in mitogen-treated preparations.

DISCUSSION

The mechanisms of the transduction of the mitogenic signals of neurally-derived mitogens are largely unknown; the roles of second messengers have just begun to be explored. Although cAMP and cholera toxin, an adenyl cyclase activator, can stimulate Schwann cell proliferation (5,6,7,10), cAMP has not been implicated as a second messenger in the transduction of the mitogenic effects of neurally-derived mitogens studied to date (5,6,11,24), with the

exception of neurites (12) in one report. We have demonstrated in this report for the first time that the mitogenic effects of axolemma and myelin-enriched fractions do not appear to be mediated by cAMP, adding to the growing list of neurally-derived mitogens that seem to promote Schwann cell proliferation by a non-cAMP route.

Evidence is also presented that suggests that calcium may act as a second messenger for the transduction of the mitogenic signals of axolemma and myelin. Removing free calcium ions by chelation with citrate (25) resulted in a profound dimunition of the mitogenic activities of both mitogens, indicating that the presence of extracellular calcium is necessary for the elaboration of their proliferative activities. The involvement of calmodulin, a calcium-binding protein, was suggested by the inhibition of the mitogenicity of both axolemma and myelin by trifluorperazine (17, 18, 19).

These tumor-promoting phorbol esters were shown to enhance the mitogenic activities of axolemma and myelin; the phorbol esters are known to influence cellular calcium metabolism at several loci (20 - 23). It is interesting to note that these esters only promoted a dramatic effect in concert with axolemma and myelin, and not when the phorbol esters were present and the mitogens absent. It is also interesting to note that axolemma preparations were consistently stimulated by PDB, but had a variable response to PMA. Myelin was consistently stimulated by both PDB and PMA.

One possible explanation of these observations is that the mitogenic expression of both axolemma and myelin may require activation of several classes of protein kinase. The biologically active phorbol esters are known to activate phospholipid-dependent protein kinases (17, 21 - 23) by replacing the endogenous cofactor (23); the intracellular phorbol receptor in some cell lines appears to actually be the protein kinase molecule (26, 27). Calmodulin is capable of activating calcium-dependent protein kinase (28). Thus, by reducing either free extracellular calcium or active calmodulin this class of protein kinase would fail to be activated and the mitogenic effect of the membranes reduced, as shown in Table I. By adding exogenous active phorbol

esters, the mitogenicity of axolemma and myelin would appear enhanced, by activation by the esters of additional phospholipid-dependent protein kinase, as seen in Table II. By triggering the activation of a protein kinases, with calcium and phospholipid serving as possible second messengers, axolemma and myelin could start a cascade that results in the stimulation of mitosis in Schwann cells.

This model may also explain why exogenous cAMP promotes Schwann cell proliferation but does not seem to increase during neurally-derived mitogen-stimulation of Schwann cell proliferation. The cellular effect of cAMP is assumed to be activation of a cAMP-sensitive protein kinase (29). It has also been suggested that cAMP-dependent protein kinases and calcium-dependent protein kinases may act on the same cellular substrates (7). By overwhelming the system with supraphysiologic doses of cAMP, as in the studies which show that cAMP is mitogenic for Schwann cells (5,6,7, 10), substrates may be activated by cAMP-dependent protein kinases that under more physiologic conditions would be activated by the calcium-dependent protein kinases instead.

We have shown for the first time that the mitogenic response of Schwann cells to axolemma and myelin-enriched fractions does not appear to be mediated by cAMP. We also provide preliminary evidence that calcium may serve as a second messenger in this system. Further investigations concerning the mechanisms of the transduction of the mitogenic signals of axolemma and myelin for Schwann cells are currently being pursued.

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