

PC12 Cells as a Source of Neurite-derived Cell Surface Mitogen, Which Stimulates Schwann Cell Division

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ABSTRACT Primary cultures of rat dorsal root ganglion Schwann cells were used to assay the efficacy of PC12 cells in stimulating Schwann cell proliferation. Co-cultures of PC12 cells and Schwann cells assayed by [³H]thymidine labeling followed by autoradiography showed proliferation of Schwann cells only where contact occurred between PC12 neurites and Schwann cells. Membranes derived from PC12 cells were shown to have many characteristics similar to membranes derived from sensory neurons; both could mimic whole cells in stimulating Schwann cell division; both were inactivated by mild heat treatment and by trypsinization, and both elevated intracellular cyclic AMP concentrations in Schwann cells 16 h after addition of membranes. We conclude that PC12 cells will be a valuable source for the isolation of the neuronal cell surface component which controls proliferation of Schwann cells during development of the peripheral nervous system.

During development of the vertebrate peripheral nervous system, several types of nerve fibers (sensory, somatic motor, and autonomic) are extended from their neuronal cell bodies. Schwann cell proliferation occurs along each of these nerve fiber types and as Schwann cells mature they provide ensheathment for the small sensory and autonomic nerve fibers and myelination for the larger sensory and the somatic motor fibers. It is known that this proliferation, as well as myelination and basal lamina formation by the Schwann cell, are triggered by signals from the growing neurites (for general review see reference 6). The dependence of Schwann cell proliferation on the neurite has the function of restricting Schwann cell proliferation to regions in which Schwann cells are required to accommodate additional neurite extension.

The development of culture systems for the preparation of pure populations of embryonic rat Schwann cells and pure populations of neurons (24) has enabled us to begin a detailed study of the initial developmental interaction between the neuron and the Schwann cell, the neurite-dependent proliferation of Schwann cells. It has been shown that rat Schwann cell cultures prepared by this method do not proliferate in the absence of added mitogen (20), and that they respond only to a limited group of mitogens. The physiologically relevant mitogen is present in the neurite membrane; Salzer et al. (21) have shown that cell-cell contact between dorsal root ganglion (DRG)¹ neurites and Schwann cells is required for stimulation

of Schwann cell division. Membranes isolated from purified DRG neurites are able to mimic the mitogenic effect of intact neurons (14, 22). The active component(s) in the neurite membrane is(are) heat- and trypsin-sensitive and therefore includes a protein component (21). In addition to neuronal membranes, Schwann cells are also stimulated to divide by a small subset of the large numbers of potential mitogens that have been assayed (cf. 17, 22). Cholera toxin is a potent Schwann cell mitogen (1, 17, 18, 21). Since cholera toxin is known to act on biological systems through activation of adenylate cyclase, cyclic AMP, and dibutyryl cyclic AMP have been added to Schwann cell cultures and weakly stimulate Schwann cell proliferation (7, 17, 21). This information raised the possibility that all Schwann cell mitogens act via a cyclic AMP-dependent pathway. However, a Schwann cell mitogen isolated from bovine pituitary and brain (a soluble 37,000-dalton protein named glial growth factor) (4) is reported not to lead to increases in cyclic AMP in Schwann cell cultures isolated from rat sciatic nerve (17).

To extend these observations and isolate the neuronal cell-surface mitogen, it is necessary to find a more abundant source of the neurite-derived Schwann cell mitogen than the neurites themselves which are available in very limited quantities (~400 μg membrane/200–300 DRG). For this reason, bovine white matter has been used as a source of central nervous system axolemmal membranes, and several laboratories have shown that these axolemmal membranes are mitogenic for Schwann cells (7, 9). These authors concur that the axolemmal mitogen is much more heat- and trypsin-resistant than the neurite mitogen (7, 9) and, whereas neurite

¹ *Abbreviations used in this paper:* DRG, dorsal root ganglion; NGF, nerve growth factor.

derived membranes stimulate the division of Schwann cells in serum-free media, the axolemmal membrane preparation is devoid of mitogenic activity in the absence of serum (7).

A permanent cell line that expresses this cell surface mitogen would be an ideal source for the purification of this molecule. In such a cell line the mitogenic molecule(s) could be metabolically labeled, and their expression could be controlled. Although previous attempts to identify such a cell line have failed (21, 22) we were intrigued by the results of Cochran and Black (8) which showed that Schwann cells could provide trophic support for PC12 cells, although interactions of the cell types did not lead to the later developmental interactions of ensheathment or basal lamina formation. We have now demonstrated the presence of a Schwann cell mitogen on the surface of the pheochromocytoma PC12 line (11). The PC12 derived mitogen has many characteristics similar to the native neurite derived mitogen. Portions of this work have been reported in an abstract (19).

MATERIALS AND METHODS

Schwann Cell Isolation: Purified Schwann cells were obtained from embryonic day 17 rats, essentially as described by Wood (24). Dorsal root ganglia were removed from the embryos, stripped of their roots and fibrous capsule, and grown on ammoniated rat tail collagen substrates (cf. 24) for 2 wk in medium C (Eagle's minimal essential media with 10% human placental serum, 6 mg/ml glucose, 2 mM glutamine, 10–20 u of nerve growth factor) with 10^{-5} M fluorodeoxyuridine to kill rapidly growing fibroblasts. Media was exchanged every 2–4 d. After 2 wk, ganglia were excised using sterile razor blade fragments, transplanted onto fresh collagen coated dishes, and allowed to grow 4–6 wk in medium C. After this time Schwann cells cover the neuritic outgrowth which extends radially from each ganglion. For use in experiments, the centrally located ganglia were excised from the cultures to allow neurites in the outgrowth to degenerate 24 h before collagenase and trypsin treatment to allow transfer of the remaining Schwann cells (7, 22). Single cell suspensions of Schwann cells were resuspended in medium C + 2.5% soybean trypsin inhibitor to inactivate residual trypsin and counted in a hemocytometer, then plated either onto growing neurites of PC12 cells in collagen-coated dishes or in Lab-Tek 8-well tissue culture chamber slides. When slides were used, each chamber was coated with 1 drop of rat tail collagen, gentle aspiration was used to remove excess collagen, and the slides ammoniated and air-dried in a laminar flow hood for at least 2 h. Approximately 10,000 Schwann cells were added to each 0.6 cm² well in 200 μ l medium C with 2.5% soybean trypsin inhibitor which was added to prevent residual trypsin from causing plated cells to lift off the collagen substrate.

Neurite Preparation: Embryonic day 15 rat DRG cultures were obtained exactly as described by Salzer et al. (21) and grown to obtain Schwann cell-free neurite growth. Isolation of membranes was modified in that whole cultures, including ganglia were used. Including ganglia increased yield of membrane/culture and did not affect the mitogenic characteristics of the membranes as compared with cultures from which ganglia were excised (21, 22). Membranes were isolated by Dounce homogenization of 8–10 cultures (10–13 DRG/culture) in 4 ml homogenization buffer (0.25 M sucrose, 0.1 mM MgCl₂, 10 mM tris pH 7.4, 17 mU/ml aprotinin). Unbroken cells, collagen and nuclei were removed by a 10-min centrifugation at 2,000 g, and crude membranes isolated from the supernatant by centrifugation for 90 min at 30,000 g. The membrane pellet was resuspended in minimal volumes of homogenization buffer by Dounce homogenization and protein concentration determined by a modification of the Lowry procedure for membrane proteins (15). All membrane fractions described in this paper were sterilized by UV irradiation for 20 min before addition to Schwann cells.

Culture of PC Cells: PC12 cells were grown either in suspension in RPMI 1640 (16), 10% horse serum (KC Biological, Lennox, KS), 5% fetal calf serum, over tissue culture plastic as described by Greene and Tischler (11), or on ammoniated collagen for 10 d with 50 ng/ml nerve growth factor (NGF) in the same media for cultures in which neurite extension was desired. In some experiments PC12 cells were grown in the N2 serum-free media (2) on collagen. Crude PC12 membranes were prepared as described for neurites except that $\sim 10^7$ cells were homogenized/ml in a 40-ml Dounce homogenizer. Modifications of the isolation procedure are described in the figure legends.

Schwann Cell Proliferation Assay: Schwann cells plated in Lab-Tek slides were assayed for incorporation of [³H]thymidine by autoradiography

as described by Cassel et al. (7) except that 1 μ Ci/ml of [³H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) was added per ml of media. Freshly prepared membrane fractions were added to Schwann cell cultures 24 h after plating of Schwann cells, and then for an additional 24 h either freshly prepared membranes or membranes frozen overnight at -80° C in homogenization buffer were added to cultures along with [³H]thymidine.

Trypsinization of Viable PC12 Cells: Cultures of PC12 cells in 125-cm² flasks were pelleted by centrifugation ($5' \times 800$ rpm) in complete media, resuspended in 50 ml Hanks' Balanced Salt Solution, repelleted, and incubated for 20 min at 37° C in 25 ml of the same solution containing 0.05% crystalline trypsin (233 U/mg). 25 mg of solid soybean trypsin inhibitor was then added to inhibit trypsin and the cells were collected by centrifugation. Cells were resuspended in cold homogenization buffer and membranes isolated. For trypsinization of membrane samples, PC12 membranes were extracted with Na₂CO₃ (see Results) and 0.7 mg of membrane protein was incubated in 0.1 ml of homogenization buffer containing 0.05% crystalline trypsin for 20 min at 37° C. The reaction was terminated with 1 mg soybean trypsin inhibitor and the membranes were added directly to Schwann cell cultures to assay their mitogenicity.

cAMP Determination: cAMP levels were determined using the cyclic AMP radioimmunoassay kit from New England Nuclear. Twenty thousand Schwann cells were plated in each well of an ammoniated collagen coated 24-well Linbro plate. The following day the media was aspirated and cells fed with 0.5 ml media containing various mitogens. All assays were carried out in triplicate. Samples were prepared for assay by removal of media, and freeze-thawing cells three times in liquid nitrogen in 0.2 ml 6% trichloroacetic acid. After pelleting of insoluble material, supernatants were extracted four times with ~ 1.5 ml water saturated ether, freeze-dried, and assayed as described in the radioimmunoassay kit, including acetylation of the samples. Cholera toxin was obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

To determine whether PC12 cells are mitogenic for Schwann cells, clumps of PC12 cells were grown for 11 d in N2 medium (2) containing 50 ng/ml NGF. The PC12 cells extended neurites and exhibited essentially no [³H]thymidine incorporation (cf. 10, 13). Schwann cells were then plated onto the PC12 cells and cultures maintained for two additional days. One μ Ci/ml of [³H]thymidine in N2 medium was then added for 24 h prior to fixation and autoradiography. Fig. 1 illustrates cell proliferation observed in these cultures. In regions where Schwann cells are in contact with PC12 neurites (B and D) many Schwann cells have incorporated [³H]thymidine into their DNA whereas in regions distant from neurites (A and C), there are no labeled cells. We also observed regions of such cultures where PC12 neurites and Schwann cells appeared to be apposed, but where Schwann cells had not been stimulated. Parallel cultures of DRG neurons and Schwann cells gave a much more homogeneous response; in all parts of these cultures, 25–50% of the added Schwann cells incorporate [³H]thymidine after a 24-h pulse of [³H]thymidine (data not shown). This experiment indicated that intact PC12 cells are mitogenic for Schwann cells, albeit to a lesser extent than DRG neurites, and that the PC12 mitogen was most likely a surface component, as is the neurite mitogen, since dividing Schwann cells were only found in the vicinity of PC12 processes.

Membranes were then isolated from PC12 cells. In initial experiments, a crude particulate fraction was isolated (see Materials and Methods) from PC12 cells grown in suspension or from PC12 cells grown on collagen in the presence of NGF and therefore containing neurite outgrowth. A very low (nearly undetectable) level of stimulation was obtained even at the highest concentrations of membranes assayed (data not shown). Further purification of the membranes was performed by collecting membranes that sediment at the top of a 1.2 M sucrose cushion for 2 h at 80,000 g. Such membranes

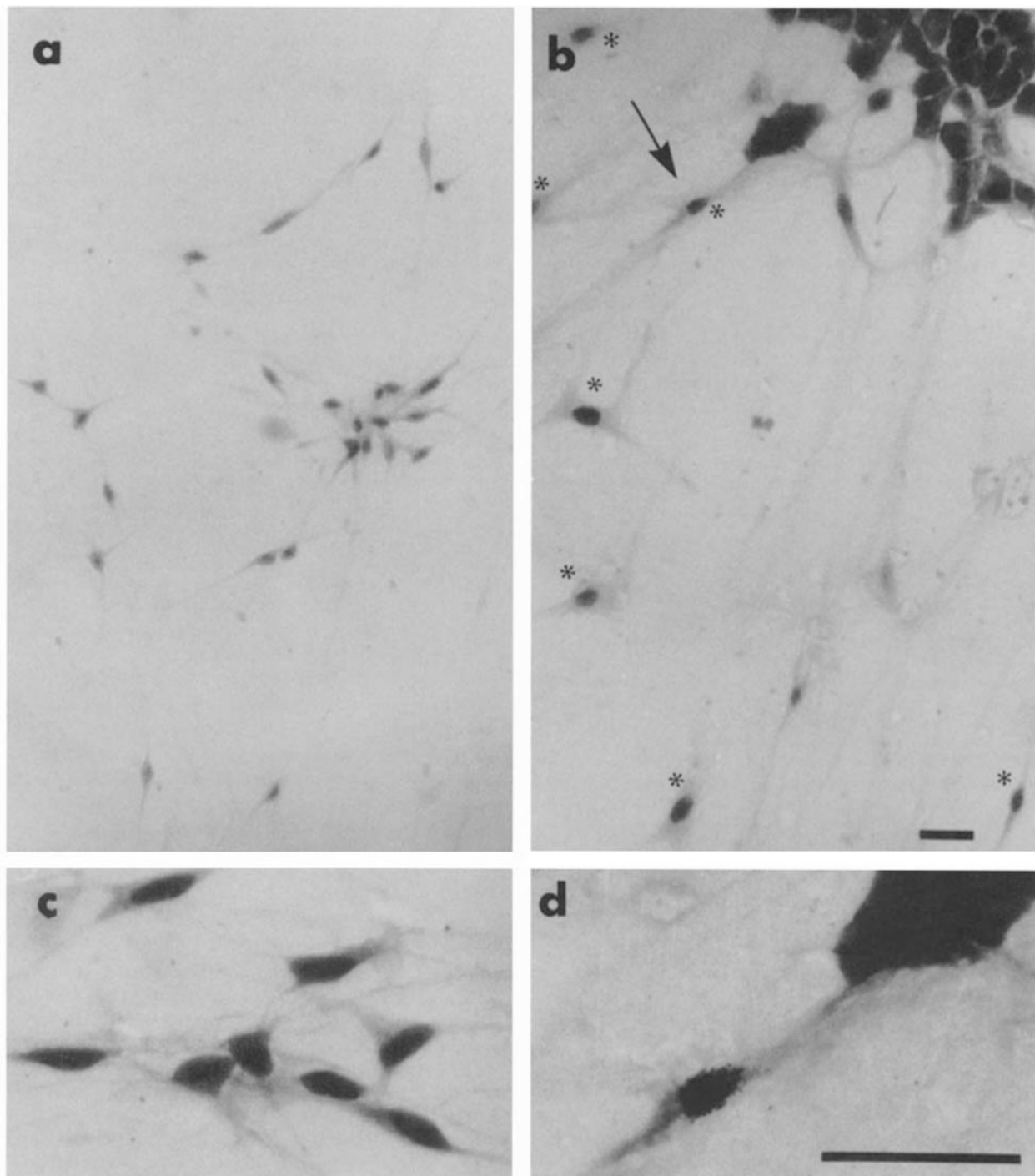


FIGURE 1 Autoradiographs of co-cultures of PC12 cells and Schwann cells in serum-free media. Experimental protocol is described in Results section. (a) Schwann cells in a section of the culture not containing PC12 cells. (b) PC12 cells (upper right) with extended neurites. Many Schwann cells on these neurites have incorporated [^3H]thymidine; these are denoted with asterisk. Grains are not in focus in order to show PC12 cells and neurites. (c) Higher magnification of Schwann cells in the absence of neurites. (d) Enlargement of Schwann cell in *b* designated by arrow. At this magnification and focal plane grains are apparent over Schwann cell nuclear region. Bar, 25 μm . $\times 333$ (a and b); $\times 864$ (c and d).

were weakly mitogenic (Fig. 2). Extraction with various agents that remove proteins weakly attached to the membranes (Table I and Fig. 2) showed that extraction with 0.2 M Na_2CO_3 (pH 10–11) resulted in a very active membrane fraction. Extraction with high salt or chelating agents did not improve the specific activity of the membrane preparation.

The Na_2CO_3 extracted PC12 membrane pellet recovered by

centrifugation in a microfuge stimulated the proliferation of Schwann cells at levels similar to those of neurite membranes (Fig. 2). The mitogen from DRG surface membrane (neurites) was also resistant to extraction with Na_2CO_3 under the conditions described for PC12 membranes, indicating that both mitogens are intrinsic membrane components (Table II). The Na_2CO_3 extraction appeared to release inhibitory material

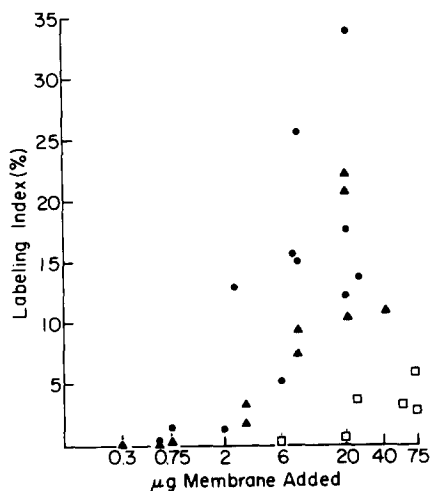


FIGURE 2 Dose-response curve of PC12 membranes before and after extraction at alkaline pH. Data shown are from five independent experiments, which accounts for the scatter in the points, since background and sensitivity of Schwann cells differ in individual experiments. Each point represents the average of duplicate samples. ●, crude neurite membranes ($n = 5$); □, crude PC12 membranes ($n = 3$); ▲, PC12- Na_2CO_3 extracted membranes ($n = 4$).

TABLE I
Removal of Peripheral Proteins from PC12 Membranes:
Correlation with Mitogenicity

Membrane fraction	Labeled cells	
	%	Yield %
No addition	1.5	
Unextracted PC12 membranes	3.1	100
0.2 M NaCl extracted PC12 membranes	5.3	44
0.2 M Na pyrophosphate extracted PC12 membranes	4.3	32
0.2 M Na_2CO_3 extracted PC12 membranes	22.8	22

PC12 membranes prepared by sucrose density centrifugation were treated with buffers that extract peripheral membrane proteins and 20 μg of the recovered membrane fraction was assayed for the mitogenic response of Schwann cells as described in the text. Approximately 1,000 cells were counted per well, numbers are averages of duplicate samples. Yield is percent of the membrane protein recovered by microfuge centrifugation of extracted membranes; only 50% of total membrane protein is recovered following microfuge centrifugation in the absence of salt solutions.

from PC12 membranes, since a 100,000 g centrifugation of the Na_2CO_3 extract from PC12 membranes sedimented material that inhibits Schwann cell proliferation induced by neurite membranes (data not shown). Whether or not such inhibitory molecules are an artifact of membrane isolation is at present not known.

The membrane preparation described above gives a very low yield of membranes. The membrane preparation method of Thom et al. (23) gives a high yield of membranes, and includes an alkaline extraction step, since cells are lysed in borate buffer at pH 10.2. Membranes prepared by this method from PC12 cells are highly mitogenic for Schwann cells (see Table II below) and 1.6 mg of membrane protein are obtained from 0.3 ml of packed PC12 cells.

To ascertain whether the mitogen was originally present on the surface of PC12 cells, we carried out experiments similar in design to those of Salzer et al. (21). PC12 cells were treated with trypsin (under conditions where 97% of the cells remained viable as judged by trypan blue exclusion) and after inactivation of the trypsin, membranes were isolated from

TABLE II
Mitogenicity of Membranes for Schwann Cells
in Serum-Free Media

Exp. no.	Membranes added	Mitogenic response	
		Serum-containing medium %	N-2 medium %
1	None	1.0	0
	Neurite membranes 8 μg	16.9	31
	Neurite membranes Na_2CO_3 extracted 5 μg	7.5	21.3
	PC-12 membranes Na_2CO_3 extracted 20 μg	16.5	0.1
2	None	1.0	0
	PC-12 Membranes 20 μg	10.5	0.1

Cells were plated on day 1 in complete media. On day 2 membranes were added in either serum-free (N2) or serum-containing (C) media. Membranes were added in the same media on day 3 along with 1 $\mu\text{Ci/ml}$ [^3H]thymidine. On day 4 cells were fixed and processed for autoradiography. Experiment 1 is representative of four separate experiments with PC12 membranes prepared by the sucrose density centrifugation and extraction with Na_2CO_3 . Neurite membranes were prepared as described in Materials and Methods. In experiment 2, membranes were isolated by the method of Thom et al. (23).

these cells and from control cultures isolated in parallel without trypsin treatment. Only if the mitogen is present on the cell surface should trypsin treatment of intact cells abolish mitogenic activity. This prediction is borne out in the data shown in Fig. 3. Thus the neurite mitogen and the PC12 derived mitogen were both trypsin-sensitive cell surface molecules. The data show that no significant quantities of a nonsurface mitogen can be detected by our assay procedure. The data in Fig. 3 also show that trypsin treatment of isolated membranes or heat treatment of these membranes abolished the mitogenic response. The sensitivity of the PC12 mitogen was similar to that of the neurite mitogen and differed from that observed for the axolemmal mitogen. The relation of the membrane-bound mitogen to the cell surface is an important criteria that could not be applied to axolemmal membranes (7, 9).

A unique feature of the Schwann cell-neuron interaction as observed in vitro was that neuron-stimulated Schwann cell proliferation is independent of serum components. As described above (Fig. 1), PC12 cells grown in N2 media were mitogenic for Schwann cells. However, membranes derived from PC12 cells both before and after Na_2CO_3 treatment and membranes isolated according to the technique of Thom et al. (23) are inactive in the absence of serum (Table II). To determine whether alkaline extraction of membranes caused loss of a component required for mitogenicity in serum-free media, crude neurite membranes were extracted with Na_2CO_3 as described for PC12 membranes, and assayed for their ability to stimulate Schwann cell proliferation in the presence and absence of serum. Neurite membranes extracted with Na_2CO_3 retained their mitogenicity in serum-free media, indicating a difference in at least one component of the mitogenic signal between neurons and PC12 cells.

Another criterion by which Schwann cell mitogens can be distinguished is by their ability to influence Schwann cell cyclic AMP concentration (7, 17, 18, 22). We have measured Schwann cell cyclic AMP levels in the presence of neurite membranes and found that cyclic AMP levels were increased,

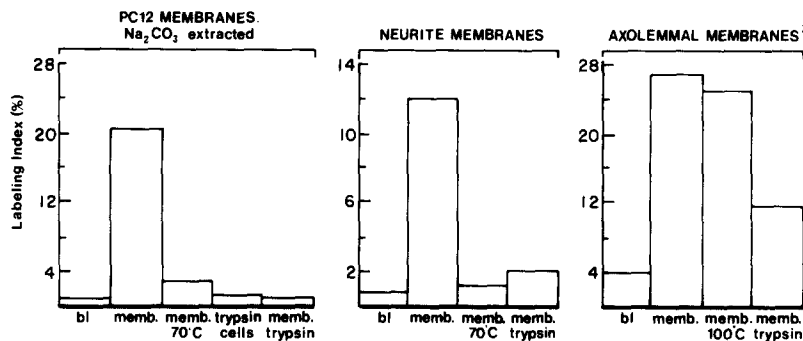


FIGURE 3 Sensitivity of membrane-bound mitogens to heat and trypsin treatment. *bl*, blank (no addition of membranes); *memb*, membranes added to cells by the protocol indicated in text; *memb 70°C* (or *100°C*), membranes treated for 10 min at the indicated temperature before addition to Schwann cells; *memb trypsin*, membranes treated with trypsin before addition to Schwann cells; *trypsin cells*, intact cells were treated with trypsin before membrane isolation and addition to Schwann cells. The data in the left panel are from the present work; the data in the other two panels are respectively, from references 21 and 9, and are presented for comparison.

TABLE III
Effect of Membrane Addition on cAMP Levels in Schwann Cells

Mitogen	Cyclic AMP*		
	Exp. 1	Exp. 2	Exp. 3
No addition	0.8	0.6	<0.5
Cholera toxin (40 ng/ml)	22.0	17.4	28.0
Neurite membranes (16 μg)	2.4	2.0	2.3
PC12 Na ₂ CO ₃ membranes (40 μg)	1.8	—	—
PC12 Na ₂ CO ₃ membranes* (40 μg)	<0.8	—	—

Schwann cells were plated in 24-well Linbro dishes on ammoniated collagen (20,000 cells/well). After 24 h mitogens were added for an additional 16 h and triplicate wells assayed for cyclic AMP by radioimmunoassay as described in Materials and Methods. The standard deviation is ~20% for all values listed.

* Picomoles per 10⁶ cells.

* Heated at 70°C for 10 min.

although to a much smaller extent than with cholera toxin. Table III shows the cyclic AMP concentration 16 h after incubation of replated Schwann cells with various mitogens. Neurite membranes increased the level of cyclic AMP in Schwann cells 3–4 times over background, whereas PC12 membranes extracted with Na₂CO₃ stimulate cyclic AMP production 2.5 times. Cholera toxin increased cAMP levels in Schwann cells 25 times over background. PC12 membranes whose mitogenic activity had been inactivated 90% by heating to 70°C for 10 min did not increase cyclic AMP levels in these cells.

DISCUSSION

We have demonstrated that a surface component of PC12 cells is similar in properties to the neuronally located Schwann cell mitogen. We have therefore overcome the major obstacle to isolation and characterization of the mitogen, the inadequate quantities of available neurite membrane.

The rat pheochromocytoma cell line, PC12, is derived from an embryonic adrenal tumor. Upon incubation with nerve growth factor, PC12 cells attach to appropriate substrata and begin to extend neurites and express neuron-specific proteins such as acetylcholinesterase, acetylcholine receptors, and choline acetyltransferase; they also undergo morphological changes such as elaboration of microtubule arrays in neurites and the appearance of synaptic vesicles (12). We anticipated that when NGF induced the conversion of PC12 cells from

chromaffin-like cells to sympathetic neuron-like cells, the membrane-bound mitogenic signal on PC12 cells would appear. In five separate experiments we found instead no difference in stimulation of Schwann cell proliferation by membranes derived from PC12 cells grown in the presence or absence of NGF (data not shown); thus, the mitogen is a constitutive component of the surface of PC12 cells.

The identification of a Schwann cell mitogen on the surface of PC12 cells will be advantageous for the isolation of the mitogen. PC12 cells can be grown in large quantities either in culture or as subcutaneous tumors in New England Deaconess Hospital rats, the strain from which the cells were originally isolated. Preliminary experiments corroborate the fact that the mitogen is present in similar quantities on PC12 cells derived from tumors and cells maintained in tissue culture.

One criteria by which the neurite mitogen and the PC12 mitogen are similar is that addition of either membrane to Schwann cells causes an increase in cyclic AMP in the Schwann cell. While such an increase has not been demonstrated previously, this result is not unexpected since cyclic AMP, dibutyryl cyclic AMP, and the phosphodiesterase inhibitor isobutylmethylxanthine all stimulate Schwann cell proliferation (18) in sciatic nerve-derived Schwann cells (3). Cyclic AMP and dibutyryl cyclic AMP have also been shown to stimulate Schwann cell proliferation in the DRG-derived Schwann cells used here (7, 22). The similarity of the Schwann cell response to neurite and PC12 cell-derived membranes using the criteria of intracellular cyclic AMP concentrations distinguishes these mitogens from glial growth factor, which appears not to elevate cyclic AMP levels in sciatic nerve-derived Schwann cells (17).

It has previously been demonstrated that neurons cultured with Schwann cells stimulate Schwann cell proliferation in serum-free media, and that membranes derived from DRG neurons are also mitogenic for Schwann cells in defined media (7). These results have been corroborated in the course of this study. Although we have demonstrated the mitogenicity of intact PC12 cells for Schwann cells when the two cell types were maintained in defined, serum-free media, membranes derived from PC12 cells (grown in the presence or absence of NGF) were not mitogenic for Schwann cells. We are led to postulate a labile component of the mitogen, as previously suggested by Cassel et al. (7) for the axolemmal mitogen, which is dissociated from PC12 cells when membranes are isolated by Dounce homogenization or by osmotic lysis of the

cells. This data distinguishes the neuronal mitogen from the PC12 cells mitogen. We are proceeding to isolate the PC12 cell-derived mitogen, however, assuming that the two systems have at least a major component(s) in common. The PC12 cell membrane-derived mitogen and the DRG neurite-derived mitogen are similar, based on their (a) sensitivity to heat, (b) sensitivity to trypsin treatment, (c) ability to stimulate intracellular cyclic AMP in Schwann cells, and (d) stability following extraction of membranes at high pH. These characteristics differentiate these mitogenic molecules from other Schwann cell mitogens.

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