Journal of Cellular Biochemistry 18:433-445 (1982)
Cellular Recognition 713-725

Mitogenicity of Brain Axolemma Membranes and Soluble Factors for Dorsal Root Ganglion Schwann Cells

Dan Cassel, Patrick M. Wood, Richard P. Bunge, and Luis Glaser

Departments of Biological Chemistry (D.C., L.G.) and Anatomy and Neurobiology (P.M.W., R.P.B.), Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Previous studies in this laboratory have shown that membranes derived from dorsal root ganglia (DRG) neurites are mitogenic for cultured Schwann cells derived from the same source [Salzer et al (1980):J Cell Biol 84:767-778]. Improved procedures are described for preparing Schwann cells derived from dorsal root ganglia that are highly responsive to various mitogens. Under these conditions, the cells respond not only to the neurite mitogen but also to pituitary extracts, dibutyryl cyclic AMP, and cholera toxin that have been shown previously to be good mitogens for Schwannn cells derived from sciatic nerve [Raff et al (1978): Cell 15:813-822], thus reconciling discrepancies in the response of these different Schwann cell preparations to mitogens. Searching for a source of membranes more suitable for biochemical characterization of the neurite mitogen, we found that bovine brain axolemma, prepared by the method of DeVries et al [(1977):Brain Res 147:339-352] is highly mitogenic for Schwann cells. The mitotic index of Schwann cells was increased by the addition of axolemma from 0.5%-2% to 30%-50% during 24-h incubation with [^{3}H]thymidine. Half maximal effect was obtained at about 0.4 μg axolemma protein per microwell containing 2-4 $\times$ 10^3 cells. The axolemma mitogen appears to be an integral membrane protein that remains bound to the membrane under various ionic conditions but can be extracted in a partially active form with deoxycholate. Like the DRG neurite mitogen, the mitogenic activity of axolemma was abolished by trypsin treatment. Unlike the neurite preparation, however, the mitogenic activity of axolemma was only partially inactivated by heat treatment (60%-70% inactivation). A significant difference between the mitogenic activity of axolemma membranes and neurite membranes is the fact that axolemma membranes fail to stimulate Schwann cell proliferation in a defined, serum-free medium (N-2), whereas neurites show significant mitogenic activity in this medium. These findings indicate a possible difference between DRG neurites and brain axolemma either in the mitogen itself or surface components responsible for recognition between the membranes and the cells.

Key words: mitogenicity, Schwann cells, axons

Received August 31, 1981; accepted September 30, 1981.

0730-2312/82/1804-0433$04.00

Interactions among neurons and supporting cells play a crucial role in the development of the functional nervous system. While in the central nervous system such interactions are not yet understood in detail, many advances have been made in the study of the processes that lead to the ensheathment of peripheral neurites by Schwann cells. The results of in vivo and in vitro experiments suggest that during development the neuron provides the Schwann cell with a signal for proliferation and subsequently for differentiation into a myelin-producing cell [1–5, reviewed in 6,7]. Tissue culture techniques have been particularly useful in investigating the effects of neurons on Schwann cells. Cultured dorsal root ganglia (DRG) provide a useful system for such investigations [3,8,9]. Ganglia from 17–20-day-old rat embryos are first plated in the presence of fluorodeoxyuridine to kill fibroblasts. When the ganglia are subsequently transferred in medium without the antimitotic agent, the outgrowth of neurites is followed by the migration to the periphery of Schwann cells, which become associated with the neurites and are actively proliferating. After several weeks in culture proliferation slows, and some of the cells initiate the production of myelin. Excision of the ganglion from an actively proliferating culture results in a rapid decline in Schwann cell proliferation following the degeneration of the neurites [8,9]. The cells are capable of resuming proliferation upon invasion by neurites from a newly transplanted ganglion [8]. A useful feature of this system stems from the recent demonstration [10,11] that the outgrowth of DRG neurites and the ensuing Schwann cell proliferation but not myelination can also take place in a defined, serum-free medium.

In a series of investigations on the role of neurites in stimulating Schwann cell proliferation, Salzer et al [9,12,13] have shown that a membrane fraction prepared from bare neurites is mitogenic for Schwann cells. Mitogenic activity of the neurite membranes was heat and trypsin sensitive. Rigorous evidence was presented for the plasma membrane localization of the neurite mitogen and the requirement for direct contact of the neurite with the Schwann cell to induce proliferation [12].

In parallel investigations Raff et al [14,15] have studied Schwann cells prepared by different methods from sciatic nerve. These preparations show a much higher background (unstimulated) proliferation rate and are stimulated to divide by addition of either dibutyryl cAMP, cholera toxin, or a protein from the pituitary gland that recently has been obtained in highly purified form by Brockes et al [16]. The quiescent Schwann cells prepared by Salzer et al [12,13] responded poorly to these mitogenic factors, but their background proliferation rate was extremely low. We now report on the design of improved conditions for preparation of dorsal root ganglion Schwann cells that retain a very low basal proliferative rate but respond effectively to all of the mitogenic compounds mentioned above.

Further biochemical characterization of the hypothetical neurite mitogen appears to require an alternative source due to the limited amount of neurites obtainable from primary cultures. A more adequate source for axons in reasonable quantity is provided by the brain axolemma preparation described by DeVries et al [17–19]. Preliminary experiments have indicated that axolemma membranes increase the incorporation of [^{3}H] thymidine into Schwann cells.* As shown in the present study, bovine and rat axolemma preparations are highly mitogenic for cultured Schwann cells. Since the relationship between the brain axolemma and the

*G.H. DeVries, J.L. Salzer, and R. P. Bunge, manuscript submitted for publication.

peripheral DRG neurite is not obvious, a comparison between the properties of the two preparations was undertaken.

METHODS

Trypsin (two times crystallized), soybean trypsin inhibitor, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), deoxycholic acid (DOC), and dibutyryl cAMP were purchased from Sigma. Cholera toxin was from Schwarz/Mann; collagenase from Worthington; Earle's minimal essential medium (MEM) from the center for Basic Cancer Research of Washington University, and [^{3}H]thymidine (6.7 Ci/mmol) from New England Nuclear. The compositions of B medium [9] and N-2 medium [20] were as described. Pituitary extract was prepared from the anterior lobes of bovine pituitaries by a modification [15] of the method described by Gospodarowicz [21]. Protein was determined by the method of Lowry et al [22] using bovine serum albumin as standard.

Preparation of Particulate Fractions From Bovine Brain White Matter

Bovine brains, obtained from a local slaughter house, were placed in plastic bags under ice. White matter excised from hemispheres was the source for all preparations and could be used either fresh or after freezing and storage at −70°C. Axolemma-enriched fraction was prepared as described by DeVries et al [15] with the exception that concentration of sucrose in the middle layer of the sucrose density gradient was adjusted to 0.85 M. After centrifugation, the membranes at the interphase of the 0.85–1.2 M sucrose layers were collected, washed with 10mM TES, pH 7.5, resuspended in the same buffer, and stored at −70°C. The yield was 5–10 mg membrane protein per 6g of white matter. On the day of an experiment, the membranes were thawed at 37°C, diluted in 10–20 volumes of MEM, collected by centrifugation at 50,000 rpm for 20 min in a Beckman 50.1 rotor, and resuspended in MEM with 10 strokes of a 1 ml, homemade Dounce homogenizer with a tight-fitting pestle to a final concentration of 1–2 mg membrane protein/ml. The same procedures were applied for the preparation of axolemma-enriched fraction from rat brain stem.

The particulate fraction of bovine white matter homogenate was prepared by mincing 5 g white matter in 200 ml 0.32 M sucrose for 1 min at the high speed of the Waring blender, followed by homogenization with ten strokes of a Dounce homogenizer with a tight-fitting pestle. The suspension was centrifuged at 27,000 rpm for 25 min in a Beckman SW 27 rotor. The pellet was suspended in 200 ml of 10 mM TES, pH 7.5, with a Dounce homogenizer and centrifuged as above. The final pellet was resuspended in the same buffer and stored at −70°C.

Myelin was prepared according to Norton and Poduslo [23]. To insure low contaminations of axolemma, both the sucrose density gradient and the low-speed centrifugation steps were carried out for one additional time. The yield of myelin was 50 mg protein per 2 g white matter.

Neurite Membrane Fraction

Membranes were prepared by the method of Salzer et al [12] from the outgrowth of dorsal root ganglia that had been maintained for 3–4 weeks in antimitotic medium containing 10 μM fluorodeoxyuridine to prevent cell proliferation [8].

Culture System

Purified populations of Schwann cells were obtained from the outgrowth of fetal rat dorsal root ganglia, essentially as previously described [8,9]. Twenty-four hours after the excision of the ganglia, cells were detached from the dish by successive treatments with collagenase (0.05%, 30 min) and trypsin (0.1%, 30 min), then washed and suspended in B medium, and plated in silastic microwells [9,12] attached to Falcon 35 mm dishes that had been coated with rat-tail collagen and exposed to ammonia vapors [24]. The dishes were prepared on the day preceding cell transfer and were treated overnight at 34°C with B medium. This treatment appears to reduce aggregation and clumping of the replated cells. Each microwell received 3–6 $\times$ 10^3 cells, about one half of which survived, yielding cell densities of 5–10 $\times$ $10^3/cm^2$.

Standard Thymidine Incorporation Experiment

The experiments, carried out in duplicate, were initiated 22–26 h after the cells were replated. The medium was withdrawn and replaced by 30 μl of a mixture containing medium A (75% Earle's MEM, 25% human placental serum (HPS) dialyzed against MEM for 24 h, 2 mM glutamine, and 100 units/ml of penicillin and 100 μg/ml of streptomycin) and the material to be tested as a mitogenic agent. Membrane preparations were always homogenized in a Dounce homogenizer before addition to the cells. An aliquot of the mixture of medium and mitogens was stored at -70°C and was thawed and a fresh addition made to the cells 20–24 h after the first addition together with 3μCi/ml [^{3}H] thymidine. Incubation was continued for another 24 h, then the microwells were washed twice with Hanks balanced salt solution containing 0.5% BSA and the cells were processed either for autoradiography or scintillation counting.

Autoradiography

Cells were fixed for 2 h with 4% formaldehyde solution in 0.1 M sodium phosphate, pH 7.2, washed with water, and coated with emulsion (NTB-2, Kodak). After exposure for three days at 4°C, the autoradiograms were developed, cells were stained for 5 min with 4% Giemsa solution in phosphate-buffered saline, washed with water, and counted to determine the fraction of labeled nuclei.

Scintillation Counting

The dishes were treated for 30 min at 4°C with 6% trichloroacetic acid, then extracted for 30 min at 37°C with 0.3 M NaOH, 2% Na_2CO_3. To reduce the background of nonspecific [^{3}H]thymidine counts obtained in the absence of cells, the alkaline extract from each microwell was transferred to a test tube containing 20 μg Salmon testis DNA solution (Sigma). One ml of 6% trichloroacetic acid was added for 5 min, and the mixture was filtered through a GF-A glass fiber (Whatman). The tubes were rinsed with 1 ml trichloroacetic acid through the filters, which were then washed with 5 ml trichloroacetic acid, 10 ml alcohol, dried, and counted in 5 ml scintillation liquid (3a70, Research Products International). Under these conditions, only 10–20 cpm were incorporated in wells that did not contain cells, and this background incorporation was not affected by any of the mitogens tested.

RESULTS

Condition for a High Sensitivity to Mitogens of Replated Schwann Cells

Salzer et al [9,12,13] recently described the mitogenic responses of Schwann cells from dorsal root ganglia. Using cells that have been replated seven days after the excision of the ganglia, these investigators found a significant response to a neurite membrane fraction but only a weak and variable response to agents which increase intracellular cAMP levels. In a search for conditions under which the cells would reveal a higher sensitivity to mitogens, we found that decreasing the time interval between the excision of the ganglia and cell transfer greatly increases the responsiveness of the replated cells to various mitogens, including cholera toxin, dibutyryl cyclic AMP, and crude preparations of a mitogen for Schwann cells present in the anterior pituitary (Table I). These substances are known as effective mitogens of Schwann cells derived from the sciatic nerve [14,15]. While the background of cells that incorporate [^{3}H]thymidine in the absence of added mitogen was slightly higher in the freshly replated cells as compared with the results of Salzer et al, this background was still very low relatively to the incorporation in the presence of mitogens (Table I). A particularly high response was obtained with cholera toxin in the presence of which up to 67% of the nuclei were labeled with [^{3}H]-thymidine during a 24-h incubation period. Note that the use of a shorter time delay between excision of the ganglia and assay of the mitogenic factors results in a much better response to all cell mitogens that we have tested, including neurites from dorsal root ganglia (compare for example Table VII and [12,13]. This important variable was not controlled in previous experiments where it was assumed that the response of quiescent Schwann cells is independent of the time period over which they had remained quiescent [12,13]. The concentration of the crude pituitary factor used in the experiment in Table I is relatively high, yet comparable to that used by Raff et al [15] who found a linear dose response (with no indication of saturation) to crude extract of anterior pituitary up to levels of 1 mg protein/ml.

DeVries et al [17–19] have recently described the preparation of axolemma-enriched membrane fraction from mammalian brain white matter. We found that axolemma from bovine brain is a potent mitogen for Schwann cells (Tables I–V, Fig. 1).

Another factor that was found to affect the mitogenic response of Schwann cells is the collagen substrate (Table II). The highest response was obtained with cells replated on ammoniated collagen. With collagen that had not been exposed to ammonia vapors but rather had been air dried for short periods, the effect of the axolemma fraction was reduced and the response to pituitary extract was abolished. Ammonia treatment of the collagen, however, is not a specific requirement since effective mitogenic responses to both axolemma and pituitary extract were also obtained with cells replated on collagen that had been air dried for long periods (18 h, Table II). The molecular basis for this effect of substratum on the mitogenic response of these cells is not known.

Properties of the Axolemma Mitogen

Figure 1 shows the dose-response relationship for the mitogenic effect of the axolemma fraction on Schwann cells. The incorporation of [^{3}H]thymidine was

TABLE I. Mitogenic Responses of Freshly Replated Schwann Cells*

Mitogen added	Time after excision of ganglia: 1 day % Labeled nuclei	7 days % Labeled nuclei
None	1.6 ± 0.8 (6)	0.8 ± 0.5 (3)
Pituitary extract (2 mg/ml)	18 ± 5 (4)	7.1 ± 5.0 (3)
Dibutyryl cAMP (0.1 mM)	49 ± 14 (4)	–
Cholera toxin (5 ng/ml)	54 ± 13 (4)	12.8 ± 0.3 (2)
Axolemma (4μg)	39 ± 6 (6)	9.7 ± 3.7 (3)

*Schwann cells were replated into microwells as described under Methods. Cells were replated either one day or seven days after excision of the ganglia. Cells were incubated with mitogens for 24 h, and then for another 24 h with fresh mitogens and in the presence of [^{3}H]thymidine, as described in Methods. After fixation, the cells were processed for autoradiography and the percentage of cells with labeled nuclei was determined. Results are the mean ± standard deviation of the number of experiments indicated in the brackets.

TABLE II. Effect of the Collagen Substrate on the Response of Schwann Cells to Axolemma and Pituitary Extract*

Collagen	% Labeled nuclei: No additions	Axolemma (4 μg)	Pituitary extract (2 mg/ml)
Air dried for 2 h	0.4	12.2	0.8
Air dried for 18 h	0.6	31.2	14.6
Ammoniated	0.9	42.9	21.5

*Conditions were as described in Methods except that collagen-coated dishes were either directly dried in a laminar flow hood or first treated with ammonia vapors and then washed with water and dried for 1.5 h.

assessed either by autoradiography or more conveniently by scintillation counting. Very similar results were obtained by both methods. The effect of axolemma was half maximal at 0.4 μg membrane protein per microwell, reached a maximum at about 4 μg and was slightly decreased at higher amounts of membranes (Fig. 1). Based on the amount of membrane protein the dose-response for axolemma is similar to that described for DRG membranes [12]. Salzer et al [13] have reported that the mitogenic activity of neurites for Schwann cells is trypsin and heat sensitive. It was of interest to test the effect of such treatments on the mitogenicity of axolemma. As shown in Table III, treatment of the axolemma fraction with trypsin almost completely abolished its mitogenicity. Soybean trypsin inhibitor prevented the effect of trypsin. Heat treatment, however, resulted in only 60%–70% decrease in the ability of axolemma to stimulate Schwann cell proliferation, even though the conditions employed were more extreme than those required to completely abolish the mitogenicity of neurites (compare Table III and ref 13). Freezing and thawing which was reported to decrease the mitogenic activity of neurites [12] does not seem to affect the mitogenicity of axolemma (not shown).

Mitogenic Activity in Brain White Matter Fractions

The preparation of axolemma according to DeVries et al [17–19] involves a sucrose density gradient step that affects the separation of axolemma from a heavy

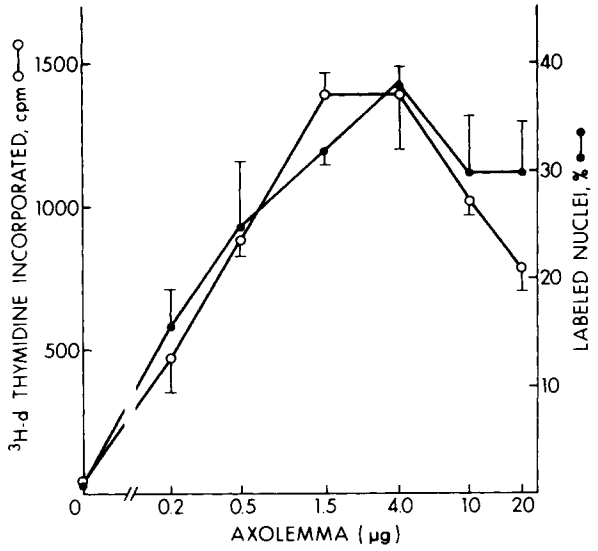

Fig. 1. Effect of axolemma on the incorporation of [^{3}H]thymidine into Schwann cells as determined by scintillation counting and autoradiography. Cells were incubated in the presence of [^{3}H]thymidine and the indicated amounts of axolemma membrane protein, and were processed for either scintillation counting (○) or autoradiography (●) as described in Methods. Bars indicate the standard deviation from the mean of quadruplicate determination.

TABLE III. Heat- and Trypsin-Sensitivity of the Axolemma Mitogen*

	[^{3}H]thymidine incorporated, CPM	% Stimulation
No membranes	65 ± 15	
Control membranes	1,700 ± 154	100
Treated membranes:		
80°C for 10 min	709 ± 110	38
100°C for 3 min	594 ± 4	32
Trypsin	173 ± 65	7
STI	1,350 ± 110	79
STI + trypsin	1,600 ± 150	94

*Axolemma membranes were suspended in MEM to give 1 mg protein/ml. Heat treatment was performed under the conditions described in the table and was followed by cooling and Dounce homogenization to affect the disaggregation of the precipitated matter. For treatment with trypsin, membranes were incubated at 30°C for 60 min in the presence of trypsin (0.15 mg/ml), soybean trypsin inhibitor (STI, 0.3 mg/ml) or both added together. Incubation with trypsin was terminated by the addition of STI to give 0.3 mg/ml. Results are expressed as the mean ± the range of duplicate determinations.

nuclear fraction and from the myelin containing fraction, which floats to the top of the gradient. The protein yield of axolemma is about 2%–4% of the total white matter, and therefore a considerable purification of mitogenic activity in the axolemma fraction might be expected. Table IV shows a comparison of the mitogenic activities of axolemma, the particular matter prepared from the whole white matter homogenate and purified myelin prepared according to Norton and Poduslo [23]. Rather unexpectedly, the mitogenic activity found in axolemma was only about 1.5 times higher than that of the whole particulate matter. On the other hand purified

TABLE IV. Effects of Bovine White Matter Fractions on Schwann Cells Proliferation*

Addition	[^{3}H]thymidine incorporated, CPM	% Stimulation relative to axolemma
None	65	–
Axolemma: 0.5 μg	694	–
4 μg	1,700	–
Whole particulate matter:		
0.5 μg	434	59
4 μg	1,100	63
Myelin: 0.5 μg	181	18
4 μg	225	10

*The different fractions were prepared as described in Methods.

myelin revealed little mitogenicity for Schwann cells. Thus, the possibility that the mitogenicity of the other fractions is due to myelin contaminations can be ruled out. The protein patterns of the fractions were compared by using SDS-polyacrylamide gel electrophoresis (Fig.2). It can be seen that myelin is a major constituent in the whole particulate homogenate, and is also present in small amounts in the axolemma preparation. Also, the particulate fraction contains a high proportion of bands similar in molecular weight to those present in the axolemma fraction; these bands are essentially absent in the myelin preparation. It is possible that the proportion of axolemma proteins in white matter is actually much larger than what might be expected from the yield obtained in the procedure of DeVries et al [17–19].

The low yield of the mitogenic activity in the axolemma fraction could be due to trapping of this material in other fractions. A total balance of the mitogenic activity could not be obtained since the precipitate formed during the three flotation steps in the DeVries procedure [17–19] are highly aggregated and cannot be tested for mitogenicity. Using saxitoxin binding as an assay for sodium channels [25], Dr. S. Goldin (Department of Pharmacology, Harvard Medical School) has found only a 3.0-fold enrichment of this axonal surface marker between crude homogenates and purified axolemma, comparable to our results.

Extraction of Mitogenic Activity With Deoxycholate

As a first step towards the isolation of the hypothetical axolemma mitogen, one needs to extract the mitogen from the membrane in an active form. The mitogen appears to be an integral membrane protein since the activity could not be removed from the membrane under conditions that are known to remove peripheral proteins, namely high or low ionic strength, chelation of divalent cations and an incubation at pH 8–10. Attempts to extract mitogenic activity with the detergents octylglucoside and cholate were unsuccessful. In preliminary experiments we have found that deoxycholate (DOC) could be successfully used to extract from axolemma material with mitogenic activity for Schwann cells (Table V). After dialysis to remove the detergent, the high-speed supernatant from DOC-treated membranes increased the incorporation of [^{3}H]thymidine into cells up to about 33% of the value observed in the presence of saturating amount of axolemma. About 40% of the membrane protein was extracted by DOC. It should be noted, however, that the effect of the DOC extract was variable. In approximately 50% of the experiments addition of the extract did not result in an increased incorporation of [^{3}H]thymidine

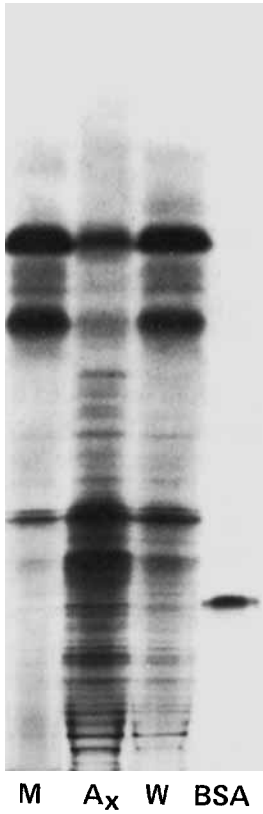

Fig. 2. Sodium dodecyl sulfate-polacrylamide gel electrophoresis of bovine white matter fractions. The fractions (50μg protein each) were subjected to electrophoresis in 13% slab acrylamide gels according to the method of Lammeli [33]. Ax = axolemma, W = whole particulate fraction, M = myelin, BSA = bovine serum albumin standard (5 μg).

TABLE V. Extraction of Mitogenic Activity From Axolemma With Deoxycholate*

Additions to Schwann cells	[^{3}H]thymidine incorporated, CPM
None	100
Axolemma (4 μg)	1900
DOC extract (6.5 μg)	700

*Six-tenths of a milliliter of axolemma membrane suspension (2 mg/ml in 10% glycerol, 0.2 mM EDTA, and 20 mM TES, pH 7.5) was incubated for 60 min in the cold with 1% recrystallized deoxycholate, with occasional shaking. After centrifugation for 1.5 h at 50 K rpm in a Beckman 50.1 rotor, the supernatant was dialyzed for 24 h against 500 volumes of 20 mM TES, pH 7.5, with one change, then for 12 h against 100 volumes of MEM.

into Schwann cells. This was apparently due to a toxic effect of the extract as judged by its inhibition of the axolemma effect as well as a pronounced shrinkage of the cells.

Mitogenic Responses in Defined Medium

In view of recent findings [10,11] that in the presence of neurons Schwann cells can actively proliferate in a defined, serum-free medium (N-2), we have investigated the response of replated Schwann cells to various mitogens in this medium. As shown in Table VI, in N-2 medium, Schwann cells showed a small response to pituitary extract and a more significant one to dibutyryl-cAMP and cholera toxin. All these responses, however, were 3–4 times lower in N-2 than in serum-containing medium (compare Tables I and VI). On the other hand, axolemma failed to induce any mitogenic response in N-2 medium. The cells did respond to axolemma when human placental serum was added to N-2 (data not shown), making it unlikely that the lack of response in the absence of serum is due to the presence in N-2 of an inhibitory substance. Table VII shows that neurite membranes derived from dorsal root ganglia are mitogenic both in the presence and absence of serum. Thus the reported ability of axons to support Schwann cell proliferation in N-2 [10,11] is preserved in membranes derived from them. On the other hand, axolemma was mitogenic only in the serum-containing medium. The response to axolemma requires a high serum concentration, 10% serum is only half as effective as the 25% serum used in all our assays. As our Schwann cells are derived from rats, the homologous preparation of axolemma from rat brain was also tested. Like the bovine axolemma, the rat preparation was active in the presence of serum but inactive in the defined N-2 medium (Table VII).

DISCUSSION

In the present study, we were able to obtain replated DRG Schwann cells with mitogenic response that are much higher than those previously reported for such cells [9,12,13]. This was achieved by shortening the time between the excision of the ganglia and the replating of the cells. The decreased proliferative response of Schwann cells that have been maintained for extended periods in the absence of axons might be related to the deep quiescent state observed in other cultured cells upon prolonged maintenance in a nonproliferative state [26,27]. Included among these observations is the finding that cells in a deep quiescent state have decreased numbers of ribosomes; this effect has been observed not only with fibroblasts [26] but also with long term quiescent Schwann cells [M. Bunge, unpublished observations]. Although basal proliferation was slightly increased in the freshly replated cells, it still remained very low. Apparently the mitogenicity of the DRG neurites decays to sufficiently low levels during the two days which elapse between excision of the ganglia and the initiation of the experiment [see ref 9], and further inactivation is caused by the trypsin treatment used in the replating procedure. It was also found that the physical form of the collagen substrate significantly affects the responsiveness of the replated cells (Table II). This is in line with findings by others [28,29]; which have recently demonstrated the remarkable effects of the substratum on the properties of cultured cells.

In this study, we have demonstrated the capability of brain axolemma to

TABLE VI. Mitogenic Responses of Schwann Cells in Defined Serum-Free Medium*

Mitogen added	% Labeled nuclei
None	0.3
Pituitary extract (2 mg/ml)	6
Dibutyryl-cAMP (0.1 mM)	12
Cholera toxin (5 ng/ml)	16
Bovine axolemma (4 μg)	0.5

*Cells were replated in a serum-containing medium (B-600) under the standard conditions. After 24 h, the medium was thoroughly withdrawn by vacuum aspiration and replaced by N-2 medium containing the indicated additions. 22 h later, the medium was withdrawn again and N-2 medium containing the same additions together with [^{3}H]thymidine added for another 24 h.

TABLE VII. Mitogenic Effects of Neurites and Axolemma in Medium With and Without Serum*

	% Labeled nuclei	
Addition	Medium with serum (A)	Serum-free medium (N-2)
Experiment I		
None	2.5	0
Neurites (2 PDE units)	49	21
Bovine axolemma (4 μg)	49	0.5
Experiment II		
None	2.2	0.15
Rat axolemma (4 μg)	25	0.5

*Conditions were as described in Table VI except that the cells received either N-2 or A medium. PDE, alkaline phosphodiesterase used as a marker for neurite membranes (see [12]).

stimulate the proliferation of cultured Schwann cell. These finds are of interest as DRG Schwann cells are only stimulated by a limited number of mitogens and not by many others, including serum and membrane preparations from several sources [9,12,15]. It is however important to consider whether the hypothetical axolemma mitogen is related to the mitogen represented by the neurite to the Schwann cell in the DRG system. The potency of axolemma membranes as a mitogen is similar to that of DRG neurites. Mitogenic activity in both preparations is trypsin sensitive, suggesting that the mitogens are proteins. However, heat treatment, which was reported to completely abolish the neurite activity [13], caused only partial decrease in the axolemma mitogenicity. Most notably the axolemma preparation failed to stimulate Schwann cell proliferation in a defined (N-2) medium, whereas neurites were remarkably mitogenic in this medium (Table VII). While these results indicate that the two membrane-bound systems are not identical, this does not necessarily indicate a difference in the mitogen itself. In view of the complexity of other cellular recognition systems [30], it is not unlikely that in addition to the mitogen axonal membranes express another type of molecules that are responsible for the proper recognition and attachment of the axonal membrane to the Schwann cell. If this is indeed the case, the difference in the effects between axolemma and neurites might also reflect a difference in a recognition unit. Clearly, further dissection of the

system will depend on the purification and characterization of the axolemma mitogenic system.

Another possibility to be considered is that the stimulation of Schwann cell proliferation by axolemma might be a nonspecific particle effect resembling the mitogenic effect of some inorganic salt precipitates as found for confluent 3T3 cells [31,32]. Unlike 3T3's, however, calcium pyrophosphate is not mitogenic for Schwann cells [unpublished observations]. Also the findings of Salzer et al [12] should be mentioned, namely that membranes from several tissue culture cells failed to stimulate Schwann cell proliferation, as well as our results that unlike axolemma brain myelin membranes are not significantly mitogenic. Finally, we have observed that axons growing from embryonic retinal explants into fascicles of Schwann cells stimulate the proliferation of the cells [P. Wood, unpublished] suggesting that CNS axons may indeed be mitogenic for Schwann cells.

Under the present conditions, pituitary extract and agents that elevate intracellular cAMP levels, previously reported to enhance the proliferation of Schwann cells derived from sciatic nerve [14,15], were also found to be mitogenic for the DRG-derived cells. The question arises whether some of the different agents that are mitogenic for Schwann cells exert their action via a common mechanism. This does not seem to be the case with cAMP and pituitary extract [14]. Also, we were unable to detect any significant binding to the axolemma of monoclonal antibody (prepared in the laboratory of Dr. J.P. Brockes) directed against the purified pituitary mitogen [D. Cassel and J.P. Brockes, unpublished observations]. The question of whether neurites stimulate Schwann cell proliferation via a cAMP mechanism has not yet been directly tested. Such a mechanism would be in accord with our observation that neurites and cAMP both retain their mitogenicity for Schwann cells in defined, serum-free medium.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Salzer for guidance in the initial phases of this work. This work was supported by grants from the National Institutes of Health GM18405, NS09923, GM28002 and from the National Science Foundation PCM7715972. D. Cassel was supported by a C. Weizmann Fellowship.

REFERENCES

1. Wood PM, Bunge RP: Nature (London) 256:662, 1975.
2. McCarthy KD, Partlow LM: Brain Res 114:415, 1976.
3. Aguayo AJ, Epps J, Charron L, Bray GM: Brain Res 104:1, 1976.
4. Aguayo AJ, Charron L, Bray GM: J Neurocytol 5:565, 1976.
5. Weinberg HJ, Spencer PS: Brain Res 113:363, 1976.
6. Spencer P, Weinberg H: In Waxman SG (ed): "Physiology and Pathobiology of Axons." New York: Raven Press, 1978, p 389.
7. Varon SS, Bunge RP: Annu Rev Neurosci 1:327, 1978.
8. Wood P: Brain Res 115:361, 1976.
9. Salzer JL, Bunge RP: J Cell Biol 84:739, 1980.
10. Manthorpe M, Skaper S, Varon S: Brain Res 196:467, 1980.
11. Moya F, Bunge MB, Bunge RP: Proc Natl Acad Sci USA 77:6902, 1980.
12. Salzer JL, Williams AK, Glaser L, Bunge RP: J Cell Biol 84:753, 1980.
13. Salzer JL, Bunge RP, Glaser L: J Cell Biol 84:767, 1980.

14. Raff MC, Hornby-Smith A, Brockes JP: Nature (London) 273:672, 1978.
15. Raff MC, Abney E, Brockes JP, Hornby-Smith A: Cell 15:813, 1978.
16. Brockes JP, Lemke GE, Balzer DR: J Biol Chem 255:8374, 1980.
17. DeVries GH, Norton WT, Raine CS: Science 175: 1370, 1972.
18. DeVries GH, Matthiue J, Beny M, Chicheportiche R, Lazdunski M, Dolivo M: Brain Res 147:339, 1978.
19. Stanley J, Saul RG, Hadfield MG, DeVries GH: Neuroscience 4:155, 1979.
20. Bottenstein JE, Sato GH: Proc Natl Acad Sci USA 76:514, 1979.
21. Gospodarowicz D: Nature (London) 249: 123, 1974.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall FJ: J Biol Chem 193:265, 1951.
23. Norton WT, Poduslo SE: J Neurochem 21:749, 1973.
24. Bornstein MB: Lab Invest 7:134, 1958.
25. Rhoden VA, Goldin SM: J Biol Chem 254:11199, 1979.
26. Baserga R: In "Multiplication and Division in Mammalian Cells." New York: Dekker, 1976, p 175.
27. Darzynkiewicz Z, Sharpless T, Staiano-Coico L, Melamed MR: Proc Natl Acad Sci USA 77:6696, 1980.
28. Gospodarowicz D, Delgado D, Voldavski I: Proc Natl Acad Sci USA 77: 4094, 1980.
29. Reid LN, Rojkind H: Methods in Enzymol 59:263, 1979.
30. Frazier W, Glaser L: Annu Rev Biochem 48:491, 1979.
31. Barnes DW, Colowick SP: Proc Natl Acad Sci USA 74:5593, 1977.
32. Rubin H, Sanui H: Proc Natl Acad Sci USA 74:5026, 1977.
33. Laemmli UK: Nature (London) 227:680, 1970.