Nonneuronal Cells Mediate Neurotrophic Action of Vasoactive Intestinal Peptide

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Abstract. The developmental regulation of neuronal survival by vasoactive intestinal peptide (VIP) was investigated in dissociated spinal cord–dorsal root ganglion (SC-DRG) cultures. Previous studies demonstrated that VIP increased neuronal survival in SC-DRG cultures when synaptic transmission was blocked with tetrodotoxin (TTX). This effect was further investigated to determine if VIP acted directly on neurons or via nonneuronal cells. For these studies, SC-DRG cells were cultured under conditions designed to provide preparations enriched for a particular cell type: astrocyte-enriched background cell (BG) cultures, meningeal fibroblast cultures, standard mixed neuron–nonneuron (STD) cultures, and neuron-enriched (N) cultures. Addition of 0.1 nM VIP to TTX-treated STD cultures for 5 d prevented the TTX-mediated death and the death that occurred naturally during development in culture, whereas the same treatment on N cultures did not prevent neuronal cell death. Conditioned medium from VIP-stimulated BG cultures prevented neuronal cell death when added to the medium (10% of total volume) of N cultures treated with TTX. The same amount of conditioned medium from BG cultures that were not treated with VIP had no protective action on N cultures. Conditioned medium from N or meningeal fibroblast cultures, either with or without VIP treatment, did not prevent TTX-mediated cell death in N test cultures. These data indicate that VIP increases the availability of neurotrophic survival-promoting substances derived from nonneuronal cultures, the most likely source being astroglial cells. This study suggests that VIP has a role in mediating a neuron–glia–neuron interaction that influences the trophic regulation of neuronal survival.

NEURONAL cell death is characteristic of most developing neural systems in vertebrates. The extent (30–80%) of neuronal death that occurs during development indicates that the regulation of this process is of fundamental importance to the determination of nervous system structure (see Berg, 1982). Although a great deal of descriptive data has been reported concerning the magnitude and ubiquity of this neuronal cell loss, little is known of the mechanism that regulates the process during development. It is clear that electrical activity plays an important role in determining neuronal survival during this regressive phase of development. For example, blockage of electrical activity with α-bungarotoxin attenuates the naturally occuring cell death in spinal motorneurons (Pittman and Oppenheim, 1978) and in trochlear nucleus in vivo (Creazzo and Sohal, 1979).

Studies with cultured spinal cord–dorsal root ganglion (SC-DRG)[1] neurons have shown that during development in vitro, neuronal cell death also occurs in a predictable and activity-dependent manner (Brenneman et al., 1985*a*). Analysis of the effects of activity blockade on neuronal survival in culture has indicated an interaction between conditioning substances and electrical activity. When endogenous conditioning substances were removed before electrical blockade, neuronal cell death was accelerated (Brenneman et al., 1983). In contrast, when conditioning substances from SC-DRG cultures were supplied during blockade of electrical activity, neuronal cell death was prevented (Brenneman et al., 1984).

The nature of these conditioning substances is unknown, as is their regulation during development. Efforts toward the identification of the substances responsible for the survival-promoting effects of conditioned medium have led to the suggestion that vasoactive intestinal peptide (VIP) can mimic the effects of conditioned media on neuronal survival (Brenneman et al., 1985*b*; Brenneman and Eiden, 1986). VIP has been shown to increase the survival of neurons during activity blockade with TTX. These survival-promoting effects of VIP were observed only at low ($<10^{-9}$ M) concentrations. This peptide has been shown to be present in dissociated SC-DRG cultures and the spontaneous release of

1. *Abbreviations used in this paper*: BG, astrocyte-enriched background; GFAP, glial fibrillary acidic protein; N, neuronal; NF, neurofilament protein; NSE, neuron-specific enolase; SC-DRG, spinal cord–dorsal root ganglion; STD, standard cultures composed of neurons and nonneuronal cells; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide.

VIP was found to be sensitive to electrical blockade with TTX. The central issue addressed in the present work pertains to the site of action for the neurotrophic effects of VIP: neuronal versus nonneuronal cells. Evidence will be presented that VIP interacts with nonneuronal cells to produce a trophic substance necessary for neuronal survival, rather than acting directly on neurons.

Materials and Methods

Cell Culture

Dissociated SC-DRG cultures were prepared from 12–14-d-old fetal mice (C57B16J) by previously described methods (Ransom et al., 1977). Cultures were plated on collagen-coated dishes. The plating medium consisted of MEM containing 10% fetal calf serum, and supplemented with $NaHCO_3$ (1.5 g/liter) and glucose (600 mg/ml). Cultures were maintained at pH 7.4 in a 10% CO_2 atmosphere at 36°C. After 24 h, the cultures were changed to one of the following media to support growth of neurons and nonneuronal cells or to enrich one or the other cell type. For standard (STD) cultures composed of neurons and nonneuronal cells, the nutrient medium consisted of a defined medium (Romijn et al., 1982) supplemented with 5% horse serum. The plating density of these cultures was 0.6 million cells per 35-mm dish. For neuronal (N) cultures that were reduced in the number of background cells, the defined medium without serum was used. The plating density of the neuronal cultures was 2.0 million cells per 35-mm dish. The STD cultures and those maintained in serum-free medium received half exchanges with fresh medium twice a week. The nutrient medium for astrocyte-enriched background (BG) cultures was 10% fetal calf serum in MEM. The plating density was 0.4 million cells per 35-mm dish, and these cultures were given a complete change of medium every 2–3 d. Fibroblast cultures were prepared from spinal cord meninges obtained from newborn mice. Meninges from five cords were treated with 0.125% trypsin for 15 min and then 15% fetal calf serum in MEM was added. After trituration, the cell suspension was added to a 75-cm^2 flask. At 10 d after plating, the cultures were confluent and the cells were treated again with trypsin, scraped from the flask, and suspended in 10% fetal calf serum/MEM. Cultures were plated at 0.2 million cells per 35-mm dish and were used as a source of conditioned medium when confluent (7 d after plating).

Conditioned medium was collected into glass bottles and stored at −20°C until used. The respective growth medium for the various cultures was used for conditioning in most experiments. The serum-free, defined medium was employed for some studies with BG cultures in order to show that the trophic effects were not dependent on the presence of 10% fetal calf serum/MEM medium. For collections of conditioned media from STD, N, and BG cultures, the age of the donor cultures was 4–6 wk. The duration of the collection period was 5 d for all donor cultures. For peptide treatment of donor cultures, the VIP was added once at the beginning of the 5-d collection period. The amount (10% of the total volume) of conditioned medium added from VIP-stimulated BG cultures was determined in preliminary experiments to be the minimum quantity that produced maximum survival activity.

Immunohistochemistry

Neuronal identity on the basis of morphology visible with phase-contrast optics was confirmed by immunohistochemical staining for neurofilament protein (NF; Dahl and Bignami, 1977) or neuron-specific enolase (NSE; Schmechel et al., 1978). Cultures were rinsed three times with MEM and fixed for 30 min in freshly prepared 4% paraformaldehyde in 0.07 M phosphate buffer (pH 7.4) at room temperature, followed by several rinses with PBS (0.01 M phosphate buffer, pH 7.4, containing 0.9% NaCl). Triton X-100 (0.1%) and normal goat serum (20%) in PBS were applied to cultures for 1 h at room temperature. After following a brief wash with PBS, the cultures were incubated overnight at 4°C with primary antibodies against NF (1:1000) or NSE (1:1000). After three rinses with PBS, the cultures were stained by the avidin–biotin–peroxidase technique (Hsu et al., 1981) using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as directed. Peroxidase was visualized by incubation in 3,3′-diaminobenzidine tetrahydrochloride (0.75 mg/ml) containing 0.01% hydrogen peroxide in 0.05 M Tris-HCl, pH 7.6. After rinsing, the cultures were osmicated (0.1% OsO_4 in Tris-HCl) for several seconds, rinsed, and stored in glycerol-Tris buffered saline containing Zephiran chloride (1:750). Controls were incubated with normal rabbit serum in place of the primary antibody; no specific staining was seen under these conditions. Cultures that were not stained for immunohistochemistry were fixed with 2.5% glutaraldehyde (60 min, 25°C) in 0.15 M sodium cacodylate (pH 7.4).

Neuronal cell counts were obtained by direct inspection of fixed cultures with a phase-contrast microscope (160×). Cultures were coded and counted without knowledge of their treatment. Neurons were counted in 100 microscopic fields (each 0.12 mm^2) from predetermined coordinate positions.

Astrocytes were identified by immunohistochemistry using antibodies against glial fibrillary acidic protein (GFAP; Dahl and Bignami, 1976). Cultures were processed as described above, except that cells were permeabilized with 0.05% saponin in PBS for 30 min, and exposed to 2% normal goat serum in PBS for 10–30 min. Primary antibody (1:5000) was applied to the cultures overnight in the cold, and GFAP immunoreactivity was visualized by the avidin–biotin–peroxidase technique as above.

VIP (Peninsula Laboratories, Inc., Belmont, CA) was dissolved in 0.01 N acetic acid as a 1-mM stock solution and stored in liquid nitrogen.

Results

Dissociated SC-DRG cultures are composed of a complex mixture of neuronal and nonneuronal cell types. Under our standard conditions, neurons grow on layers of nonneuronal background cells. Immunohistochemical methods were used to identify and quantify the total number of neurons and astrocytes. For these studies, NF and NSE were used as neuronal markers and GFAP as the astrocytic marker. In each experiment neurons from each treatment group, identified as phase-bright cells with multiple branching processes, were counted. In addition, NSE- or NF-immunoreactive cells were counted in some experiments. Stained and unstained cultures are shown in Fig. 1.

To identify the target cell population for the action of VIP, the cellular composition of the cultures was experimentally manipulated by changing the type of nutrient medium, modifying plating densities, and adjusting feeding schedules. With these techniques, the relative number of neurons versus nonneuronal cells could be varied. The two types of test cultures are shown in Fig. 1. The STD cell cultures were maintained in MEM supplemented with 5% horse serum and defined medium components. N cultures grown in serum-free defined medium contained a larger proportion of neurons relative to astrocytes, and were considered neuron-enriched. A comparison of counts of both neuronal and GFAP-positive cells is shown in Table I.

Growing the cultures under serum-free conditions reduced the number of neurons by 40%, whereas the number of GFAP-positive cells was reduced by ~60%. The numbers of surviving neurons in these cultures were compared after electrical blockade and/or VIP treatment (Fig. 2). In both types of cultures, treatment with tetrodotoxin (TTX) produced similar percentage deficits from the respective controls, and treatment with VIP, in the absence of TTX, resulted in little change from the neuronal counts of control cultures. In contrast, the effect of VIP and TTX co-treatment on neuronal counts was greatly different between the two types of cultures. Standard cultures exhibited counts after VIP/TTX treatment that were not significantly different from those observed in control cultures at the beginning of the test period (day 14). Therefore, in STD cultures, the TTX-mediated neuronal cell loss, and the loss of neurons that occurs naturally between 2 and 3 wk in culture, apparently were prevented by VIP/TTX treatment. The same experimental treatment in N cultures maintained under serum-free conditions did not produce these neuron-sparing effects. Signifi-

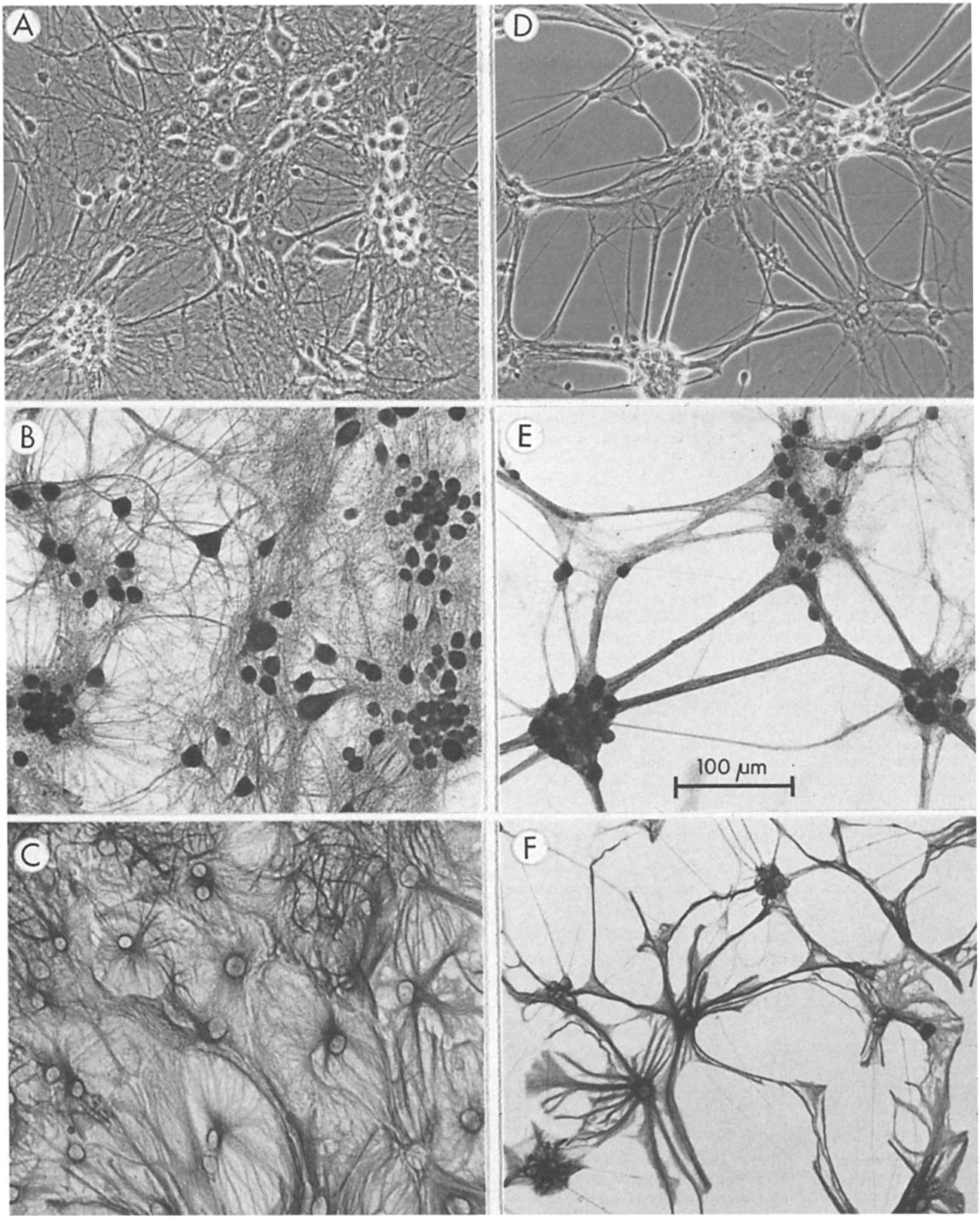

Figure 1. Dissociated SC-DRG cultures, 3 wk in vitro, maintained in serum-containing (*A–C*) or serum-free (*D–F*) medium. With phase-contrast optics (*A* and *D*), the serum-supplemented cultures appear to contain a higher density of neurons, which exist both in aggregates and in loose networks. In the serum-free cultures, most of the neuronal cell bodies are aggregated and the neuronal processes are highly fasiculated. Similar cultures were stained immunohistochemically using antibodies against NSE (*B* and *E*) and GFAP (*C* and *F*) to visualize neurons and astrocytes, respectively. Staining for NSE confirms that the phase-bright processed cells are neurons, and that the neurons tend to aggregate more strongly when grown in serum-free medium. Staining for GFAP demonstrates that, in the serum-supplemented cultures, a large proportion of the culture surface is covered by GFAP-immunoreactive cells. The serum-free cultures contain far fewer GFAP-positive cells and lack the nearly confluent astrocyte substratum. Antiserum against NSE was generously provided by Dr. P. Marangos; that against GFAP, by Dr. D. Dahl.

Table I. Comparison of Cell Counts on SC-DRG Cultures: Effect of Nutrient Medium

Culture medium	Neurons (NSE +)	Astrocytes (GFAP +)
5% Horse serum and defined medium	896 ± 46	773 ± 34
Defined medium	525 ± 20	328 ± 11
Defined medium/serum	59%	42%

Cells were counted in 100 fields. Cultures were 21 d old. Each value is the mean ± the SEM of three culture dishes.

cant decreases in the numbers of neurons after treatment with VIP and TTX were not different from those observed with TTX treatment alone. Therefore, VIP appeared to have no survival-promoting effects in cultures maintained under serum-free conditions. These data suggest that the effects of VIP may be related to the cellular composition of the test cultures, i.e., the presence and density of astrocytes. To obtain direct evidence for this possibility, the cellular (i.e., neuronal, glial, or fibroblast) site of action for the neurotrophic effects of VIP was investigated by using three types of cultures as donors of conditioned medium (Fig. 3). Donor BG cultures maintained in 10% fetal calf serum in MEM were composed predominantly of nonneuronal cell types. Whereas there was an abundance of GFAP-positive material in these cultures, there were very few neuronal cells evident from the NF staining. Counts of the NF-positive cells indicated that <0.1% of the neurons remained as compared with STD cultures. Donor cultures maintained in serum-free medium exhibited abundant NF-positive cells and greatly reduced numbers of GFAP-positive cells compared with BG donor cultures. For these experiments, VIP (0.1 nM) was added to both types of donor cultures. Conditioned medium was collected from the VIP-treated donors and also from similar cultures that had not received VIP stimulation. Meningeal fibroblast cultures were used similarly as a source of fibroblast-conditioned medium. This was done as a control for the presence of fibroblasts in BG donor cultures that displayed considerable fibronectin immunoreactivity (data not shown). The remainder of the studies employed N test cultures for evaluating the various conditioned media for trophic activity. The characteristic of the N test cultures that was exploited was their lack of response to the neurotrophic effects of exogenous VIP coupled with their responsiveness to trophic substance present in conditioned medium from BG cultures. Conditioned media from the donor cultures described above were compared for their efficacy in preventing the loss of neurons during electrical blockade with TTX (Fig. 4). Treatment with TTX alone produced a significant decrease in the number of neurons. Addition of conditioned medium (10% of total volume) from BG cell cultures (as shown in Fig. 3, *A* and *B*) produced no effect as compared with controls. The same BG-conditioned medium added to TTX-treated test cultures produced no detectable protection from the neuronal deficits produced by TTX. In contrast, TTX-treated test cultures that received conditioned medium from BG donor cultures treated with 0.1 nM VIP showed a significant sparing of neurons; there was no significant decrease in the number of neurons as compared with control cultures at the beginning of the test period (day 14). The survival-promoting effect of BG-conditioned medium was observed when either 10% fetal calf serum/MEM or serum-free, defined medium was used for conditioning. Treatment of electrically active N test cultures with conditioned medium from VIP-stimulated BG cultures did not have a significant effect on the number of neurons as compared with control cultures. Treatment of test cultures with VIP or VIP plus TTX confirmed the previous observation that VIP itself was not effective in preventing TTX-mediated cell death in the test cultures maintained in serum-free medium. Since VIP treatment was not effective in providing protection in this test system, the sparing effect appeared to be due to a substance other than VIP.

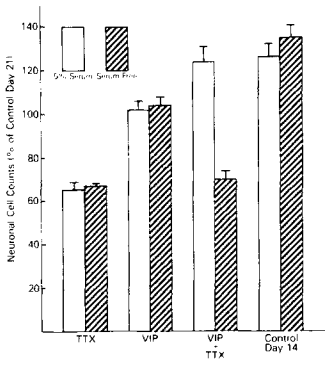

Figure 2. Neuronal cell counts in serum-supplemented and serum-free cultures after treatment with VIP (0.1 nM) or VIP plus TTX. Test cultures were maintained in 5% horse serum/MEM with defined medium components (*open bars*) or in serum-free defined medium (*hatched bars*). Cultures were treated on day 14 after plating and then fixed for cell counting on day 21. The plating density was one million cells per 35-mm dish. Control cultures maintained in standard serum-containing medium had neuronal cell counts of 1422 ± 56 per 100 fields at the end of the test period. Sister cultures grown in serum-free defined medium had significantly ($P < 0.001$) fewer neurons (1025 ± 40). Statistical comparisons of neuron counts for cultures in serum-containing medium revealed: control day 14 = VIP plus TTX > control day 21 = VIP > TTX ($P < 0.001$). Neuron count comparison for cultures maintained in serum-free defined medium: control day 14 > control day 21 = VIP > VIP plus TTX = TTX ($P < 0.001$).

As shown in Fig. 4, conditioned medium from BG cultures not treated with VIP had no effect, at 10% total volume, on neuronal survival in TTX-treated N test cultures. However, when this same medium was provided to the test cultures at 30% total volume, significant neuron sparing (90% of control) was observed (data not shown). These data suggest a low level spontaneous release of the survival factor(s) from BG cultures and the augmentation of this release by the presence of VIP.

Additional experiments were performed to establish that VIP acted in the presence of nonneuronal cells. VIP was added to conditioned medium obtained from non-VIP-treated BG cultures and this mixture was added to TTX-treated N cultures. This mixture was 10% of the medium volume. Under these conditions, there was no evidence of protection from TTX-mediated neuronal deficits (data not shown). These data indicate that VIP must be in contact with the BG cultures to produce the survival-promoting effects.

Since the nonneuronal BG cell cultures generally contained a few neurons, additional experiments were performed to determine whether the few remaining neurons might be responsible for the conditioned medium effects. To test this, high density N donor cultures (Fig. 3, *C* and *D*) were used as a source of conditioned medium. The comparison was made between conditioned media collected from N donor cultures grown in the presence or absence of VIP (Fig. 5). Conditioned medium from N cultures did not prevent

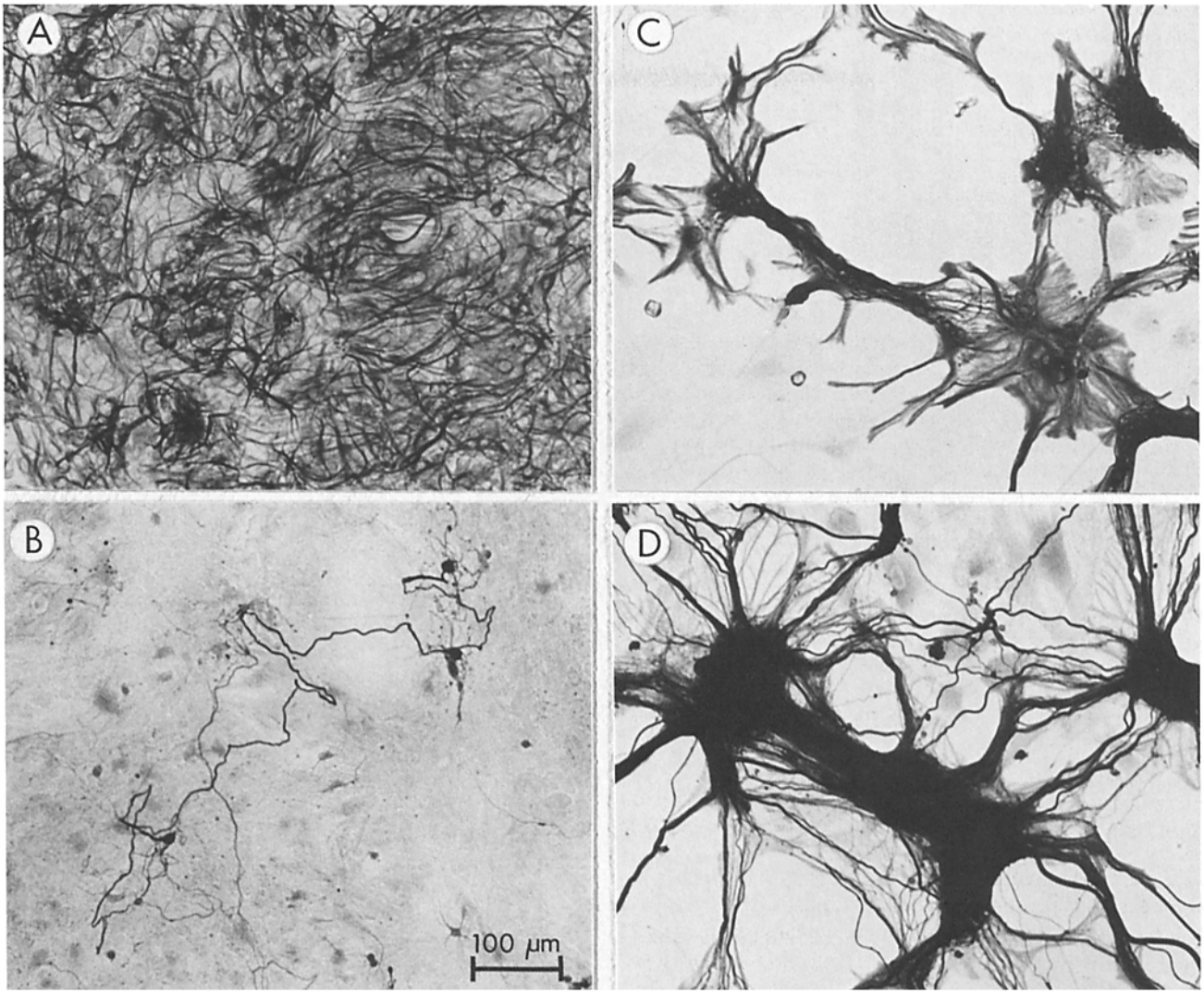

Figure 3. SC-DRG cultures maintained for 5–7 wk in 10% fetal calf serum (*A* and *B*) or in serum-free defined medium (*C* and *D*). Immunohistochemistry using antibodies against GFAP (*A* and *C*) or NF (*B* and *D*) demonstrated the preponderance of astrocytes and the scarcity of neurons in cultures grown in fetal calf serum and, conversely, a sparse astrocyte background layer and dominant neuronal aggregates in cultures grown in serum-free medium. The plating density of the cultures in 10% fetal calf serum was 0.3 × 10^6 cells/35 mm dish; and in serum-free medium, 2 × 10^6 cells/dish. Antiserum against NF was generously provided by Dr. D. Dahl.

TTX-mediated neuronal cell loss either with or without treatment of the donor cultures with VIP. The amount of conditioned medium was 30% of the total volume, a quantity three times that used for BG-conditioned medium. Sister cultures treated with conditioned medium from VIP-treated BG cultures confirmed that the test system could respond.

The BG cultures are composed predominantly of astroglia, fibroblasts, and possibly Schwann cells. To determine whether fibroblasts were involved in the production of the survival factor, meningeal fibroblast cultures, with and without VIP treatment, were used as donors of conditioned medium. These cultures were shown to be free of GFAP-positive cells. Addition of conditioned medium from fibroblast cultures (5–50% of the total volume) resulted in no detectable increase in neuronal survival in TTX-treated N test cultures. The possible contribution of Schwann cells was tested using conditioned medium from VIP-treated DRG background cultures. These cultures were grown for 1 mo in 10% fetal calf serum/MEM and then changed to serum-free defined medium for a 5-d collection period in the presence of VIP. This conditioned medium (20% of total volume) did not exhibit significant survival-promoting activity on spinal cord neurons in N test cultures (data not shown).

Previous work indicated that conditioned medium from dissociated SC-DRG cultures contained substances that could prevent neuronal cell death during electrical blockade. The conditioned medium was collected from STD cultures during days 1–6, when cultures have been shown to release substances that can prevent TTX-mediated neuronal cell death (Brenneman et al., 1984). The conditioned medium was tested on STD cultures during days 9–14, when some neurons were vulnerable to TTX-mediated death and when the release of neuron survival-promoting substances was low (Brenneman et al., 1984). Exposure of cultures to TTX decreased the number of neurons by 35%, and the conditioned medium prevented this loss of neurons. The effect of dialysis

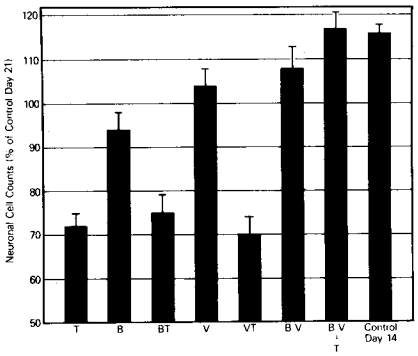

Figure 4. Neuronal cell counts in serum-free N test cultures after treatment with conditioned medium from background cell cultures: effect of VIP on donor cultures. The nonneuronal donor cultures were maintained in 10% fetal calf serum in MEM. The N test cultures were grown in serum-free defined medium. For these studies, TTX, VIP, and/or conditioned medium (200 μl with 1.8 ml of nutrient medium) was added to the test cultures on day 14 after plating and the cultures were fixed for counting on day 21. The test cultures received the following treatments: T, TTX (1 μM); B, conditioned medium from nonneuronal background cell cultures; BT, conditioned medium (as in B) plus TTX; V, VIP (0.1 nM); VT, VIP plus TTX; B-V, conditioned medium obtained from nonneuronal cultures treated for 5 d with 0.1 nM VIP; and B-V + T, conditioned medium from VIP-treated nonneuronal cultures plus TTX. Statistical comparisons: control day 14 = B-V + T = B-V > control day 21 = V = B > BT = T = VT ($P < 0.025$). Neuronal cell counts for control cultures on day 21 were 509 ± 15 per 100 fields. Each value is the mean of six to eight determinations from two experiments. The error bar is the SEM.

on these conditioned medium substances is shown in Table II. Dialysis did not appear to reduce the neurotrophic activity of the conditioned medium. The molecular weight of VIP (3326 D) was below the ~10,000-D cutoff of the dialysis membrane. These data further suggest that the neuronal survival factor is not VIP, and is a higher molecular weight, nondialyzable molecule.

Discussion

Glia are thought to have supportive roles for developing neurons by providing directional cues (Wessels et al., 1980; Silver et al., 1982; Noble et al., 1984) and growth-stimulating substances (Banker, 1980; Muller and Seifert, 1984; Eagleson et al., 1985). The substances responsible for these functions and their mechanisms of action remain to be characterized. The present study has demonstrated that a neuropeptide may serve a paracrine function between developing neurons and glia. A diffusible substance that exhibits neuronal survival-promoting activity is detectable after treatment of nonneuronal cultures with low concentrations of VIP. The cellular interaction suggested by these observations involves a neuron–glia–neuron relationship. VIP-containing neurons release the peptide during electrical activity. The peptide stimulates a population of glia to increase the production of substance(s) that are necessary for neuronal survival. The glia-derived substances apparently are used or competed for by neurons in a manner which is dependent on electrical activity. Several characteristics of the dissociated spinal cord system allow for the demonstration of VIP neurotrophic action. Of principal importance is the presence of many cell types in these primary cultures. The advantage of such complexity is apparent when screening compounds that may mediate cellular communication between unidentified populations of cells. Cellular diversity permits a variety of cellular interactions that may be required to reveal the neurotrophic action of VIP.

Of equal importance is the ability to manipulate the cellular composition of the cultures to allow for some reduction in this complexity; specifically, one may enrich the cultures for either neurons or nonneuronal cells (McCarthy and Partlow, 1976). This enrichment of cell types permitted us to address the key issue: does VIP act directly on neurons or indirectly through glia to produce its neurotrophic effects? An essential aspect of this manipulation is the identification of

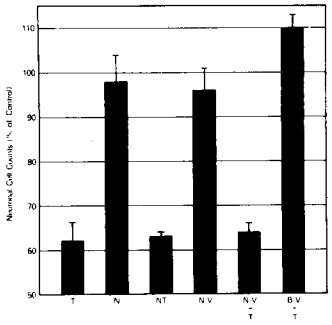

Figure 5. Neuronal cell counts in serum-free N test cultures after treatment with conditioned medium from serum-free neuronal cultures: lack of a VIP effect on donor cultures. These experiments are analogous to those presented in Fig. 4 except that the ratio of neurons to astrocytes is much greater than in donor cultures maintained in 10% fetal calf serum. Donor cultures were grown in serum-free medium for 6 wk before the collection period. For those donor cultures treated with VIP (0.1 nM), the peptide was added once at the beginning of the 5-d collection period. Test cultures, maintained in serum-free defined medium and similar to those described in Fig. 4, were treated from day 14 to day 21 with the following: T, TTX; N, conditioned medium from serum-free neuron-rich cultures; NT, conditioned medium (as in N) plus TTX; N-V, conditioned from VIP-treated neuronal cultures; N-V + T, conditioned from VIP-treated neuronal cultures plus TTX; B-V + T, conditioned medium from VIP-treated nonneuronal SC-DRG cultures plus TTX. Statistical comparisons: B-V + T = control day 21 = N = N-V > T = NT = N-V + T. ($P < 0.001$). Neuronal cell counts of control cultures were 422 ± 12 per 100 fields. All conditioned media were 30% of the total incubation volume. Each value is the mean of four to six counts; the error bar is the SEM.

Table II. Effect of Dialysis on Neuronal Survival-promoting Substances in Conditioned Medium from SC-DRG Cultures

Treatment	Neuron counts (percent of control)
TTX	65 ± 5
CM	98 ± 4
CM plus TTX	98 ± 3
CM (dialyzed)	95 ± 3
CM (dialyzed) plus TTX	96 ± 3

Conditioned medium (CM) was obtained from dissociated SC-DRG cultures after the first week in culture and frozen until used. Dialysis was performed for 24 h with three changes against phosphate-buffered saline. The molecular weight cutoff of the dialysis membrane was ~10,000 D. After dialysis, the conditioned medium was passed through a 0.22-μm filter. The conditioned medium was added to the test cultures on day 9 and the treatment period was terminated on day 14. A complete change of medium was performed before the test period. Conditioned medium was 50% of the volume of the nutrient medium. The plating density was 3×10^5 cells/35-mm dish. Neuron counts (per 100 fields) for the control cultures were 956 ± 34 (SEM).

the cell types by immunohistochemistry. The relative abundance of neurons and glia can be assessed in the various preparations. The nonneuronal cell types responsible for VIP action and the types of neurons that respond to the glia-derived substance released by VIP are yet to be identified. Experiments with meningeal fibroblasts and DRG background cells suggest that these are not involved in the production of the VIP-related neurotrophic substance. The implication of these data is that the other most abundant background cell type, the astroglia, is the source of the survival factor. Functional VIP receptors have been reported to exist in primary astrocyte cultures (Magistretti et al., 1983; Evans et al., 1984). Further reduction of the complexity of the primary cultures or utilization of astroglial cell lines will be necessary to address the next level of cellular specificity that relates to the action of VIP.

The cell culture preparation employed in the present work contained neurons derived from the spinal cord and from dorsal root ganglia. Previous studies have indicated important differences between these two groups of neurons. Nerve cells derived from DRG do not exhibit spontaneous electrical activity in culture and their survival is not influenced by electrical blockade (Bergey et al., 1981). In contrast, spinal cord neurons invariably display such activity and some of these neurons are dependent on activity for survival (Brenneman et al., 1983). The trophic regulation of these two groups was also confirmed to be different. Preliminary studies have indicated that nerve growth factor is not the glia-derived factor being studied in these experiments (data not shown). Antiserum to nerve growth factor did not block the neurotrophic effects on spinal cord neurons. Furthermore, exogenous nerve growth factor, while increasing the size and survival of DRG neurons, did not increase the survival of spinal cord neurons in the presence or absence of TTX.

Regardless of which support cell is involved in this action, our studies indicate that VIP does not produce its survival-promoting action by a direct effect on neurons. Demonstration of the neurotrophic action of VIP is dependent on the type of medium used to maintain the dissociated SC-DRG cells. TTX-treated cultures grown in defined medium do not respond to VIP, whereas this same medium supplemented with 5% horse serum allows for the expression of the trophic effects of VIP. The reason for this dependency is not certain. However, this observation coupled with the effects of conditioned medium from nonneuronal cultures suggests that the increased number of astrocytes present in cultures maintained in serum-containing medium may have a significant role in mediating this effect. Alternatively, a change in the health or function of the remaining astrocytes in the serum-free N cultures might cause them to be unresponsive to VIP. Such a change in function may be associated with the shift in astrocyte morphology that was observed in the N cultures as compared with STD cultures. The number of flattened, nonprocessed astrocytes is greatly reduced in N test cultures, whereas this type of astrocyte is abundant in STD cultures (Fig. 1, *C* and *F*).

The action of VIP on neuronal survival is evident only during electrical blockade with TTX. Several activity-dependent processes could be contributing to this effect. The first relates to the presence of endogenous VIP, and a second to the possible competition for and/or utilization of trophic substances by neurons. VIP has been shown to be present in dissociated SC-DRG cultures (Brenneman et al., 1985). Significant release of VIP occurs spontaneously; however, addition of TTX prevents this liberation of peptide. However, this does not entirely explain the dependency of neuronal survival on the peptide effects. Addition of TTX plus VIP appears to prevent neuronal cell death that results from treatment with TTX and also neuronal cell death that occurs naturally during development in culture. This conclusion is based on the observation that the number of neurons after treatment with VIP and TTX exceeds that in control cultures at the end of the test period. Since the neurons are postmitotic, the explanation for the increase in the number of neurons above that of controls is that death that normally occurs in electrically active cultures is prevented by VIP/TTX treatment. Our hypothesis is that the glia-derived substance that is stimulated by VIP is in some way competed for or used by neurons in an activity-dependent manner. Blockade of electrical activity perhaps decreases these competitive processes to allow for the survival of most neurons within the test period.

We propose that VIP stimulates the release of trophic agents important for neuronal survival. Previous studies have indicated that VIP can act as a modulator of hormone release. VIP has been shown to stimulate prolactin release by a direct action on the pituitary (Ruberg et al., 1978). In addition, VIP has been reported to modify the release of growth hormone (Vijayan et al., 1979), luteinizing hormone–releasing factor (Samson et al., 1981), and somatostatin (Epelbaum et al., 1979). Although some of these effects may not involve a direct action of VIP, the participation of VIP in the regulation of some hormones undoubtedly occurs. It is of interest that growth hormone–releasing factor (Guillemin et al., 1982) exhibits considerable sequence homology with VIP. Another explanation for the neurotrophic action of VIP is the possibility that this peptide is a glial mitogen, acting to increase the number of glia that spontaneously release neurotrophic survival factors. Further experiments are needed to resolve the releasing factor versus mitogen hypotheses.

Our study has presented evidence that indicates that the neurotrophic action of VIP, which is evident in electrically blocked spinal cord neurons, is mediated through a nonneuronal cell. This work supports the idea that glia respond to neuronal signals by secreting substances that are important to neuronal survival and development. Furthermore, we suggest that the VIP-mediated effects are a model for neuronal input on glial function, as communication between neurons and glia probably involves many substances, including other neuropeptides and neurotransmitters.

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References

Banker, G. A. 1980. Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science (Wash. DC).* 209:809–810.

Berg, D. K. 1982. Cell death in neuronal development: regulation by trophic

factor. *In* Neuronal Development. N. C. Spitzer, editor. Plenum Publishing Corp., New York. 297–331.
Bergey, G. K., S. C. Fitzgerald, B. K. Schrier, and P. G. Nelson. 1981. Neuronal maturation in mammalian cell culture is dependent on spontaneous electrical activity. *Brain Res.* 207:49–58.
Brenneman, D. E., and L. E. Eiden. 1986. Vasoactive intestinal peptide and electrical activity influence neuronal survival. *Proc. Natl. Acad. Sci. USA.* 83:1159–1162.
Brenneman, D. E., L. E. Eiden, and R. E. Siegel. 1985*a*. Neurotrophic action of VIP on spinal cord cultures. *Peptides (NY).* 6:35–39.
Brenneman, D. E., S. Fitzgerald, and M. J. Litzinger. 1985*b*. Neuronal survival during electrical blockade is increased by 8-bromo cyclic adenosine 3′,5′ monophosphate. *J. Pharmacol. Exp. Ther.* 233:402–408.
Brenneman, D. E., S. Fitzgerald, and P. G. Nelson. 1984. Interaction between trophic action and electrical activity in spinal cord cultures. *Dev. Brain Res.* 15:211–217.
Brenneman, D. E., E. A. Neale, W. H. Habig, L. M. Bowers, and P. G. Nelson. 1983. Developmental and neurochemical specificity of neuronal deficits produced by electrical impulse blockade in dissociated spinal cord cultures. *Dev. Brain Res.* 9:13–27.
Creazzo, T. L., and G. S. Sohal. 1979. Effects of chronic injections of α-bungarotoxin on embryonic cell death. *Exp. Neurol.* 66:135–145.
Dahl, D., and A. Bignami. 1976. Immunogenic properties of the glial fibrillary acidic protein. *Brain Res.* 116:150–157.
Dahl, D., and A. Bignami. 1977. Preparation of antisera to neurofilament protein from chicken brain and human sciatic nerve. *J. Comp. Neurol.* 176: 645–657.
Eagleson, K. L., T. R. Raju, and M. R. Bennett. 1985. Motoneurone survival is induced by immature astrocytes from developing avian spinal cord. *Dev. Brain Res.* 17:95–104.
Epelbaum, J., L. Tapia-Arancibia, J. Besson, W. H. Rotsztejn, and C. Kordon. 1979. Vasoactive intestinal peptide inhibits release of somatostatin from hypothalamus in vitro. *Eur. J. Pharmacol.* 58:493–495.
Evans, T., K. D. McCarthy, and T. K. Harden. 1984. Regulation of cyclic AMP accumulation by peptide hormone receptors in immunocytochemically defined astroglial cells. *J. Neurochem.* 43:131–138.
Guillemin, R., P. Brazeau, P. Bohlen, R. Esch, N. Ling, and W. B. Wehrenberg. 1982. Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. *Science (Wash. DC).* 218:585–587.
Hsu, S. M., L. Raine, and H. Fanger. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29:577–580.
Magistretti, P. J., M. Manthorpe, F. E. Bloom, and S. Varon. 1983. Functional receptors for vasoactive intestinal polypeptide in cultured astroglia from neonatal rat brain. *Regul. Pept.* 6:71–80.
McCarthy, K. D., and L. M. Partlow. 1976. Preparation of pure neuronal and non-neuronal cultures from embryonic chick sympathetic ganglia: a new method based on both differential cell adhesiveness and the formation of homotypic neuronal aggregates. *Brain Res.* 114:391–414.
Muller, H. W., S. Beckh, and W. Seifert. 1984. Neurotrophic factor for central neurons. *Proc. Natl. Acad. Sci. USA.* 81:1248–1252.
Noble, M., J. Fok-Seang, and J. Cohen. 1984. Glia are a unique substrate for the in vitro growth of central nervous system neurons. *J. Neurosci.* 4:1892–1903.
Pittman, R. H., and R. W. Oppenheim. 1978. Neuromuscular blockade increases motoneurone survival during normal cell death in the chick embryo. *Nature (Lond.).* 271:364–366.
Ransom, B. R., E. A. Neale, M. Henkart, P. N. Bullock, and P. G. Nelson. 1977. Mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties. *J. Neurophysiol.* 40:1132–1150.
Romijn, H. J., A. M. M. C. Habets, M. T. Mud, and P. S. Wolters. 1982. Nerve outgrowth, synatogenesis and bioelectric activity in fetal rat cerebral cortex tissue cultured in serum-free, chemically defined medium. *Dev. Brain Res.* 2:583–589.
Ruberg, M., W. H. Rotsztejn, S. Arancibia, J. Besson, and A. Enjalbert. 1978. Stimulation of prolactin release by vasoactive intestinal peptide. *Eur. J. Pharmacol.* 51:319–320.
Samson, W. K., K. P. Burton, J. P. Reeves, and S. M. McCann. 1981. Vasoactive intestinal peptide stimulates LHRH release from median eminence synaptosomes. *Regul. Pept.* 2:253–264.
Schmechel, D., P. J. Marangos, A. P. Zis, M. Brightman, and F. K. Goodwin. 1978. Brain enolases as specific markers of neuronal and glial cells. *Science (Wash. DC).* 199:313–315.
Silver, J., S. E. Lorenz, D. Wahlsten, and J. Coughlin. 1982. Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies, *in vivo*, on the role of preformed glial pathways. *J. Comp. Neurol.* 210:10–29.
Vijayan, E., W. K. Samson, S. I. Said, and S. M. McCann. 1979. Vasoactive intestinal peptide: evidence for a hypothalamic site of action to release growth hormone, luteinizing hormone and prolactin in conscious ovariectomized rats. *Endocrinology.* 104:53–57.
Wessells, N. K., P. C. Letourneau, R. P. Nuttall, K. Luduena-Anderson, and J. M. Geiduschek. 1980. Responses to cell contacts between growth cones, neurites, and ganglionic non-neuronal cells. *J. Neurocytol.* 9:647–664.