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Vasoactive intestinal peptide and electrical activity influence neuronal survival

(neuronal death/development/tetrodotoxin/tetanus toxin/cell culture)

DOUGLAS E. BRENNEMAN*† AND LEE E. EIDEN‡

*Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, and ‡Laboratory of Cell Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20205

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ABSTRACT **Blockade of electrical activity in dissociated spinal cord cultures results in a significant loss of neurons during a critical period in development. Decreases in neuronal cell numbers and ^{125}I-labeled tetanus toxin fixation produced by electrical blockade with tetrodotoxin (TTX) were prevented by addition of vasoactive intestinal peptide (VIP) to the nutrient medium. The most effective concentration of VIP was 0.1 nM. At higher concentrations, the survival-enhancing effect of VIP on TTX-treated cultures was attenuated. Addition of the peptide alone had no significant effect on neuronal cell counts or tetanus toxin fixation. With the same experimental conditions, two closely related peptides, PHI-27 (peptide, histidyl-isoleucine amide) and secretin, were found not to increase the number of neurons in TTX-treated cultures. Interference with VIP action by VIP antiserum resulted in neuronal losses that were not significantly different from those observed after TTX treatment. VIP_{10-28}, a fragment that inhibits VIP stimulation of adenylate cyclase, also produced a dose-dependent decrease in neuronal cell counts similar to that seen with TTX treatment. These data indicate that under conditions of electrical blockade a neurotrophic action of VIP on neuronal survival can be demonstrated.**

Most neural systems of mammalian embryos are comprised of significantly more neurons than are present at maturity (1). The regulation of the cell death that accounts for this paradoxical change during development is not understood. However, spontaneous electrical activity (2–4) and trophic substances (5, 6) have been shown to influence neuronal cell death and have been hypothesized to be involved in a mechanism by which function determines the number of neurons that comprise a mature neural network.

The relationship between electrical activity and trophic activity is only beginning to be explored, and it may be that there are multiple types of interaction. For example, spontaneous electrical activity may be linked to a mechanism by which neurons compete for trophic substances. Activity in this case may promote neuronal death by eliminating redundant neurons on the basis of their relative ability to obtain such trophic support. Evidence that supports this aspect of activity include observations that electrical blockade increases neuronal survival during developmental periods when neuronal death is normally occurring. Studies *in vivo* (3, 4) and with dissociated spinal cord cultures (7) support the existence of such a mechanism. In addition, a trophic substance or perhaps a trophic factor-releasing substance may be released during electrical activity. Activity in this case has the effect of promoting neuronal survival by stimulating the release of substances important for survival. In the present study, the possibility is examined that neuropeptides may be among the activity-dependent components that can influence neuronal survival during development.

Numerous neuropeptides have been found in the developing nervous system. However, their role during development is not known. We have investigated several closely related peptides for possible trophic function in developing spinal cord cultures. Vasoactive intestinal peptide (VIP) was assessed for neurotrophic action because of the following observations. VIP has been shown to be present in the spinal cord (8, 9) and in some dorsal root ganglion cells (10). Previous work has shown that VIP immunoreactive neurons are present in spinal cord cultures and that its release is prevented by tetrodotoxin (TTX) (11). Analogues of cyclic AMP increased neuronal survival in spinal cultures that were electrically blocked (12). Of the peptides that are known to be present in the spinal cord, VIP appears to be one of the most efficacious in stimulating adenylate cyclase (13, 14) in non-neuronal cells. In addition, VIP has been shown to act as a releasing agent (15, 16) or a modulator of release for other hormones (17). PHI-27 and secretin were also examined for neurotrophic properties because of their close sequence homology (18) with VIP.

Spinal cord cultures have been used as a model system to study the relationship between electrical activity and neurotrophic substances that influence neuronal survival during development (7). Some neurons die within a predictable period during development *in vitro* (12). Electrical blockade influences neuronal survival, but this effect appears related to endogenous conditioning substances that fluctuate with development (7). Blockade of electrical activity with TTX in the absence of conditioning factors produces neuronal death, whereas if such substances are supplied, an increase in neuronal survival over controls is observed (7). The latter condition results in increased survival that appears to parallel observations made *in vivo* (3, 4). Our working hypothesis is that VIP or a VIP-like molecule is an activity-dependent mediator for the release of conditioning factors that promote neuronal survival.

METHODS

Cell Culture. Dissociated spinal cord/dorsal root ganglia (SC/DRG) cultures from 12- to 14-day-old fetal mice (C57BL6J) were prepared as described (19). Cultures were plated on collagen-coated 35-mm dishes at a plating density of 6×10^5 cells per dish. Cells were grown in a medium consisting of 80% (vol/vol) Eagle's minimal essential medium (MEM), 10% (vol/vol) heat-inactivated horse serum, 10% (vol/vol) fetal calf serum (GIBCO), and supplemented with $NaHCO_3$ (1.5 g/liter) and glucose (6 mg/ml). Cultures were

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Abbreviations: TTX, tetrodotoxin; VIP, vasoactive intestinal peptide.

†To whom reprint requests should be addressed at: National Institutes of Health, Building 36, Room 2A21, Bethesda, MD 20205.

maintained at pH 7.4 in a 10% CO_2/90% air atmosphere at 36°C. The nutrient medium was changed to a medium (20) containing 5% (vol/vol) horse serum 24 hr after plating with subsequent changes made twice a week. Dissociated spinal cord cultures are a complex mixture of many neuronal cell types that grow on top of a layer of nonneuronal cells consisting of fibroblasts and various types of glia. Cell division was suppressed with 5-fluoro-2'-deoxyuridine (15 μg/ml) plus uridine (35 μg/ml) on days 5–7 after plating. Prior to treatment of the cultures with peptides or TTX, medium was completely changed to remove conditioning substances that can increase neuronal survival during electrical blockade (7). Peptides were obtained from Peninsula Laboratories (San Carlos, CA) and TTX from Sigma.

Electrical activity was blocked with 1 μM TTX. All spontaneous action potentials were absent with this treatment, and no evoked excitatory postsynaptic potentials (EPSPs) were detected (2). Whole cell patch recordings conducted on spinal cord neurons have shown that, from 24 hr after plating to 3 weeks *in vitro*, all elicited and spontaneous action potentials were TTX sensitive (21). Although no other pharmacological action of TTX has been demonstrated using the concentrations of TTX employed in the present study, the possibility of other TTX-sensitive processes being involved in these effects does exist. However, xylocaine, a local anesthetic, has also been shown to produce neuronal deficits similar to TTX (2).

Neuronal Cell Counts. Neuronal cell counts were made directly on the stage of an inverted phase contrast microscope at a magnification of 160×. Neurons were counted in 100 regularly spaced fields (0.12 mm^2) at predetermined coordinate locations. Cells with phase-bright somata and branching processes were counted as neurons. Cultures were coded and counted without knowledge of their treatment. ^{125}I-labeled tetanus toxin autoradiography (2) and whole cell patch clamp recordings (22) were employed to confirm neuronal identity of similar morphologies in sister cultures. Cells were fixed with 2.5% (vol/vol) glutaraldehyde (60 min, 25°C) followed by 0.15 M sodium cacodylate (pH 7.4) prior to counting.

^{125}I-Labeled Tetanus Toxin Fixation. ^{125}I-labeled tetanus toxin fixation was used as a neuronal surface marker (23, 24). This assay provided a rapid estimate of changes in the size and/or number of neurons after a given treatment. It was used to complement neuronal cell counts. Specific ^{125}I-labeled tetanus toxin fixation was not detected in 3-week-old, neuron-free cultures from spinal cord/dorsal root ganglia. With the conditions employed in the present study, the binding of tetanus toxin to a type of astroglia (25) appeared not to be quantitatively significant. Thus, the tetanus fixation assay in this culture system appeared to be a good neuronal marker. Preparation of the labeled toxin (26) and assay conditions (2) have been described in detail. Briefly, labeled toxin (0.2 nM; 50–100,000 cpm) was incubated with washed cultures for 60 min at 36°C. An average of 5% of the available toxin became associated with the cultures. Nonspecific fixation was determined by 1 hr preincubation with 20 nM unlabeled toxin in a parallel series of cultures and then incubation with the labeled toxin. Specific fixation varied from 55–75% of the total radioactivity associated with the cultures. To terminate, cultures were rinsed three times with phosphate-buffered saline. Cultures were dissolved in 1.5 ml of 0.2 M NaOH with aliquots taken for protein (27) and ^{125}I measurements.

RESULTS

A dose–response study of VIP-mediated effects on neuronal cell counts is shown in Fig. 1. Cultures in standard growth medium or in medium containing 1 μM TTX were treated with increasing concentrations of VIP. Treatment was started

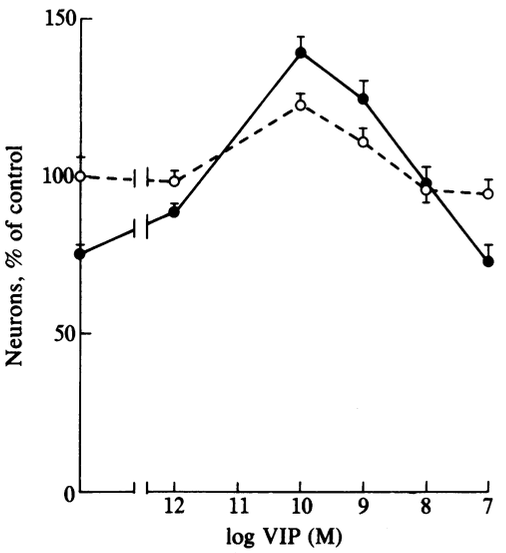

FIG. 1. Neuronal cell counts from cultures treated with VIP alone (○) or VIP plus TTX (●). Dose–response to VIP was studied in cultures that were electrically active and in cultures that were electrically blocked with TTX. Additions to the cultures were made on day 9 of culture. Nutrient medium was completely changed prior to the various treatments. After 5 days of exposure to the peptide, cultures were fixed with 2.5% (vol/vol) glutaraldehyde (60 min, 25°C) followed by 0.15 M sodium cacodylate (pH 7.4). Cell counts for day-14 control cultures were between 1500 and 2000 cells per 100 fields. Each point is the mean of six to eight determinations from two experiments. The error bar is the SEM. Statistical comparisons for all experiments were made with an analysis of variance with Student–Newman–Kuel multiple comparison of means.

on day 9 and continued until day 14, at which time cells were fixed, and the counts were conducted. Blockade of electrical activity with TTX resulted in a significant decrease ($P < 0.02$) in neuronal cell counts to 75% of day 14 controls. Addition of 0.1 nM VIP to electrically blocked cultures produced the highest number of neurons, which was greater ($P < 0.05$) than in cultures treated only with VIP. At the conclusion of the test period on day 14, the number of neurons observed after TTX plus VIP (0.1 nM) treatment was not significantly different from that observed for control cultures on day 9, the beginning of the test period. The apparent increase in cell counts after VIP treatment alone was not statistically different from day 14 control values. Assuming no VIP-mediated changes in cellular phenotype, the nominal interpretation of this effect is that VIP treatment of electrically quiescent cultures prevented neuron death that was mediated by TTX as well as death that occurred naturally during culture maturation.

Spinal cord cultures are comprised of many neuronal cell types that vary considerably in the complexity and size of their dendritic arborizations. Due to the morphological diversity of this system, a neuronal surface marker, ^{125}I-labeled tetanus toxin fixation, was used to obtain additional information about the response of neurons to electrical blockade and/or VIP treatment (Fig. 2). For these studies, the application of VIP and TTX to the cultures was made according to the same paradigm described for the experiments in which neuronal cell counts were conducted. In TTX-treated cultures, addition of VIP produced a concentration-dependent increase in ^{125}I-labeled tetanus toxin fixation as compared to those receiving TTX alone. No significant changes from control were observed after treatment with VIP alone. The general shape of the concentration–effect curve was similar to that observed for neuronal counts (Fig. 1). Thus, measurements of neuronal counts and ^{125}I-labeled tetanus toxin fixation support the conclusion that VIP can mediate neuro-

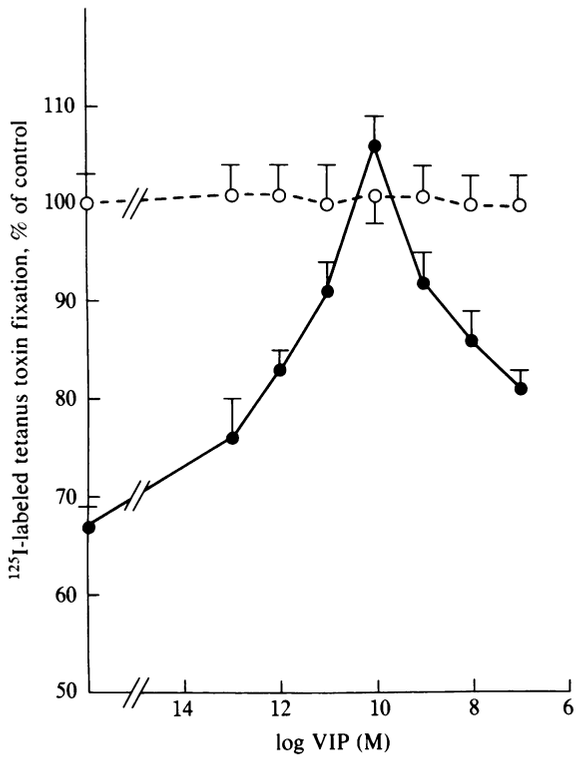

FIG. 2. ^{125}I-labeled tetanus toxin fixation of cultures treated with VIP (○) or VIP plus TTX (●). Dose–response to VIP was studied in cultures that were electrically active and in cultures that were electrically blocked with TTX. Control cultures had values that ranged from 2100–5300 cpm per culture well. Each point is the mean of 12 determinations from three experiments derived from separate dissections. The error bar is the SEM.

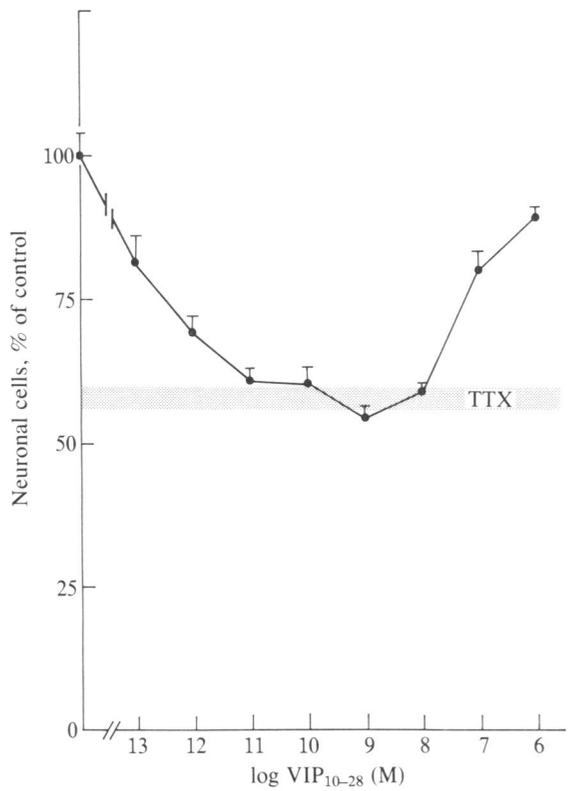

FIG. 3. Neuronal cell counts after chronic treatment with VIP_{10-28}. This carboxyl-terminal fragment of VIP has been shown to inhibit the VIP-stimulated cyclic AMP increase (28). Various concentrations of this VIP_{10-28} were added to the cultures 24 hr after plating. Treatment was continued until day 13 when the cells were fixed for counting. The shaded area indicates the cell counts from cultures treated with TTX alone in sister cultures during the same test period. Each point is the mean of four to six culture dishes. The error bar is the SEM.

trophic survival effects on a population of developing neurons grown in culture.

To further test the possibility that VIP or a VIP-like substance can influence activity-dependent neuronal survival, cell counts were conducted on cultures after treatment with a C-terminal fragment of VIP (Fig. 3). This fragment has been shown to inhibit VIP-stimulated increases in cyclic AMP (28). With increasing amounts of the VIP fragment, the number of neurons progressively decreased. The VIP fragment was very potent with 0.1 pM VIP_{10-28} producing a significant decrease ($P < 0.01$) from controls. At 10 pM, the number of surviving neurons was not significantly different from that observed after TTX treatment alone. At higher concentrations (0.1 μM and above), the increases in neuron death were attenuated.

Additional experiments with VIP antiserum were performed to test if interference with VIP function resulted in effects similar to electrical blockade. The effect of VIP antiserum on neuronal cell counts is shown in Table 1. Addition of rabbit anti-VIP antiserum produced a similar decrease in the number of neurons as that observed with TTX treatment. Simultaneous treatment with VIP antiserum and TTX did not result in an additive effect. This observation is consistent with the hypothesis that TTX and VIP act within the same mechanism that affects neuronal cell number. Normal rabbit serum alone had no effect on neuronal cell counts as compared to controls. Together, the effects of exogenous VIP, VIP_{10-28}, and VIP antiserum on neuronal cell counts all support the conclusion that VIP can influence the survival of activity-dependent neurons during development in culture.

The specificity of the VIP effects on neuronal survival was investigated by testing two closely related peptides, PHI-27 and secretin, for similar effects. Using the same experimental paradigm, treatment with concentrations of peptide ranging from 1 pM to 0.1 μM plus TTX was found not to increase the number of neurons over that observed with TTX treatment alone.

DISCUSSION

Neuronal cell death associated with electrical blockade of spinal cord neurons in culture was attenuated after treatment with low concentrations of VIP. Addition of VIP to electrically active cultures had no significant effect on cell counts and no detectable effect on ^{125}I-labeled tetanus toxin fixation. Interference with VIP action by treatment with VIP antiserum or with VIP_{10-28} caused a decrease in the number of

Table 1. Comparison of VIP antiserum and tetrodotoxin on neuronal death

	Neurons per 100 fields	% of control
Control	1132 ± 38	100
VIP antiserum	890 ± 14*	79 ± 1*
TTX, 1 μM	902 ± 24*	80 ± 2*
VIP antiserum and TTX	859 ± 16*	76 ± 2*
Normal rabbit serum, 0.25%	1145 ± 62	101 ± 5

Values are the mean ± SEM; $n = 4$. Sufficient antiserum (29) was added to bind 300 pg of VIP. Treatment was started 24 hr after plating. Cultures were fixed after 12 days of treatment.
*Significantly different ($P < 0.01$) from control.

neurons similar to that observed with the blockade of electrical activity by TTX. We attribute these effects to the release of endogenous VIP during electrical activity and hypothesize that VIP plays a vital role in the determination of neuronal survival during a critical period in development.

The effects of VIP on neuronal survival are contingent on two experimental manipulations of the culture system: the blockade of electrical activity and the removal of conditioned medium at the beginning of the test period. The reason for these obligatory changes in the culture system is not clear but these effects may relate to activity-dependent release of peptides and activity-related competition that can affect neuronal survival. If endogenous neurotrophic material is not removed, sufficient endogenous VIP and/or other substances may prevent any TTX-related decreases in the number of neurons. If activity is not blocked we hypothesize that no peptide effects were observed because of the continued effective release of endogenous VIP.

In the present study, it appeared that virtually all neurons that were vulnerable to TTX-mediated deficits were affected by VIP treatment. Evidence that supports this conclusion was the nonadditive effects of VIP antiserum and TTX treatment. In addition, the similar effects of VIP_{10-28} and TTX on neuronal cell death also are consistent with this conclusion. In our model system, the anatomical constraints have been removed by tissue dissociation and the neuronal networks that form are based on unknown preferences that may or may not relate to networks *in vivo*. We speculate that the VIP effects on neuronal survival do have relevance to events *in vivo* but that these effects are probably limited by anatomical constraints *in vivo*, thereby making this mechanism of more localized importance than the general effects suggested by the present study. Other activity-dependent networks may utilize different molecular signals to mediate survival effects.

The effects of VIP on neuronal development have been concluded to be due to survival-related changes. Several alternative explanations for decreases in the number of neurons have been considered. Treatment with VIP in the presence of TTX could produce an increase in neurons by stimulating neuronal mitosis or differentiation of precursor cell types to neurons. Although neither of these possibilities can be unequivocally rejected, several lines of evidence suggest that these explanations are unlikely. *In vivo* studies conducted on mouse spinal cord have shown that neuronal cell division was complete by day 14 gestation (30). This time corresponds approximately to the time of dissection, and thus it is 9 days before the test period employed in the present study. In addition, the spinal cord cultures were treated with antimitotic agents making the contribution of neuronal division to our observations improbable. The possibility that VIP in the presence of TTX alters differentiation of precursor cells to account for the observed increases in neuronal number has also been considered. Such an effect would have to exactly compensate for the loss of neurons produced by TTX and the loss that occurred naturally during the course of development. Such a large compensatory effect by another population of cells seems less likely than the possibility that the combination of VIP and TTX are rescuing the same cells that fail to survive in controls and the neurons that are lost by treatment with TTX alone.

VIP increases neuronal cell number by a mechanism that has not been determined. The most likely explanation is that increased neuronal survival, resulting from competition for trophic substances, is influenced by both VIP and electrical activity. It may be that the VIP is acting directly on neurons or perhaps indirectly via glial cells. Although neither of these alternatives can be excluded, several lines of evidence allow for the possibility of a glia-mediated mechanism. Functional VIP receptors have been observed on cultured astrocytes (31) and substances that promote the survival of neurons have been shown to be present in conditioned media from glial cultures (32–34). We propose that neurons release VIP in an activity-dependent manner and that VIP then interacts with glial cell receptors. We hypothesize that glial cells stimulated by VIP produce additional factors that are important for neuronal development and survival. In this scheme, VIP would act as a releasing factor.

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