Phenotypic plasticity in adult sympathetic neurons: Changes in neuropeptide expression in organ culture
(neuropeptide Y/peptide histidine isoleucine amide/secretin–glucagon family/sympathetic ganglion/vasoactive intestinal peptide)


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ABSTRACT Vasoactive intestinal peptide (VIP)-like immunoreactivity is present at low levels in the superior cervical ganglion of the adult rat, where immunostained neural processes, but only an occasional immunostained cell body, are found. However, when ganglia are maintained for 24 or 48 hr in organ culture, their content of VIP-like immunoreactivity increases 6- or 31-fold, respectively. When examined at 24 hr, the increase in VIP-like immunoreactivity is totally blocked by an inhibitor of RNA or protein synthesis. Many neuronal cell bodies and processes with immunoreactivity for VIP and the related peptide, somatostatin (PPI) are seen in cultured ganglia. In addition, VIP/PHI mRNA is abundant in cultured ganglia but only barely detectable in ganglia prior to culture. Under these culture conditions, neuropeptide Y-like immunoreactivity increases to a small extent, and tyrosine hydroxylase activity and total protein remain unchanged. These results support the idea that adult sympathetic neurons exhibit plasticity in neuropeptide expression and that this plasticity, in the case of VIP, depends on changes in gene expression.

Specific neurons can synthesize and release more than one neurotransmitter. For example, many adult sympathetic neurons synthesize and release both norepinephrine and neuropeptide Y (NPY), a small number synthesize and release acetylcholine and vasoactive intestinal peptide (VIP), and some use other combinations of amines and peptides (1). In addition, during development, neurons can change the neurotransmitters they utilize. The best studied example is the ability of neonatal sympathetic neurons to start making acetylcholine and to reduce their synthesis of norepinephrine. Initially identified in cell culture, this phenomenon was subsequently shown to occur in vivo, during the development of the cholinergic sympathetic neurons that innervate the rat foot pad (2).

In addition to changes in biogenic amine synthesis, the expression of colocalized neuropeptides in neonatal sympathetic neurons can also be regulated during development. Medium conditioned by cultured heart cells contains a factor that, in addition to producing a cholinergic “switch” in neonatal sympathetic neurons from the superior cervical ganglion (SCG), also increases their immunoreactivity for several peptides [i.e., VIP, substance P, somatostatin, but not NPY (3)]. Also, when sympathetic neurons innervating the foot pads in neonatal rats change from a noradrenergic to a cholinergic phenotype, they acquire immunoreactivity for VIP and calcitonin gene-related peptide (2). Finally, placing neonatal SCG in explant culture for 48 hr produces a large (50-fold) increase in the expression of substance P-like immunoreactivity (IR) (4).

Much less attention has been paid to the possibility that alterations in transmitter phenotype occur in adult sympathetic neurons. With electrophysiological techniques, only a small percentage of adult sympathetic neurons were found to acquire cholinergic properties in cell culture (5). Biochemical experiments in which choline acetyltransferase was measured in organ-cultured SCG also suggested a decrease in the phenotypic plasticity of these neurons with age (6). Adult SCG placed in organ culture, however, increase their substance P-IR, though with a somewhat slower time course than do neonatal ganglia (7).

MATERIALS AND METHODS

Organ Culture. SCG were removed from adult Sprague-Dawley rats (200-250 g), desheathed, and maintained in organ culture. Details of the procedure were as previously presented (8), except that BGG, medium (GIBCO BRL) was used. Ten percent newborn calf serum normally was added to the medium; however, in one experiment, a chemically defined medium was used [Ham’s F12 supplemented with transferrin (100 μg/ml), putrescine (100 μM), insulin (5 μg/ml), progesterone (20 nM), bovine serum albumin (dilipidized; 5 mg/ml), selenium (30 nM), penicillin (50 units/ml), and streptomycin sulfate (50 μg/ml)]. In certain experiments, either nerve growth factor (NGF, 100 ng/ml; Collaborative Research) or actinomycin D (1 μg/ml; Sigma) or anisomycin (100 μM; Sigma) were added to the BGG medium, and the ganglia were cultured for 24 hr. In all experiments, SCG were removed from culture after 24 hr (referred to as t = 24) or 48 hr (t = 48) and then either frozen at −80°C or immersion-fixed. In one experiment, a group of ganglia was included that were not cultured but instead were frozen or immersion-fixed immediately after being dissected from animals (t = 0).

Radioimmunoassays for VIP and NPY. Peptides were extracted from individual ganglia by boiling them in 2 M acetic acid for 20 min. An aliquot was removed for protein determination, and the remainder of the supernatant was lyophilized and then reconstituted in assay buffer containing 0.1 M sodium phosphate (pH 7.3), 0.05 M NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100, 1 mM EDTA, 0.01% Na2SO4, and aprotinin (500 kallikrein inhibitory units/ml). Reconstituted extracts of SCG and various concentrations of synthetic VIP (Bachem) were incubated for 18–24 hr with a rabbit anti-VIP antiserum (Peninsula Laboratories; final dilution, 1:90,000).

125I-VIP (NEN Research Products) was added, and the incubation was continued for an additional 24 hr. The antigen-antibody complexes were precipitated with goat anti-

Abbreviations: IR, like immunoreactivity; NGF, nerve growth factor; NPY, neuropeptide Y; PHI, peptide histidine isoleucine amide; SCG, superior cervical ganglion (ganglia); VIP, vasoactive intestinal peptide.

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rabbit IgG and normal rabbit serum and were collected by centrifugation for measurement of radioactivity in a gamma counter. The sensitivity of the assay (detection limit, ~1 pg per tube) allowed the measurement of VIP-IR in individual ganglia. NPY-IR was radioimmunoassayed (kit from Peninsula Laboratories) with a detection limit of 12 pg per tube.

The HPLC retention time of VIP-IR extracted from a ganglion that had been cultured for 48 hr was compared with that of synthetic VIP. The extract and synthetic VIP were passed through Waters SepPak C18 cartridges prior to injection onto a Vydac C18 column. Reverse-phase liquid chromatography was performed with a linear gradient of 20–50% acetonitrile in 0.1% trifluoroacetic acid for 30 min at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, dried on a Speed Vac concentrator (Savant), and assayed for VIP-IR.

Peptide Immunohistochemistry. Ganglia taken immediately after removal from animals or after 48 hr in organ culture were immersed in Zamboni’s fixative for 16–24 hr at 4°C (four to six ganglia for each experimental group for both antisera studied). The tissue was processed for the detection of VIP-IR as described by (9, 10). The VIP antiserum (from J. Allen, University of Cambridge; used at a 1:1000 dilution) and the peptide histidine isoleucine amide (PHI) antiserum (from J. Fahrenkrug, Bispebjerg Hospital, Copenhagen; used at a 1:2000 dilution) have been characterized (11, 12).

Tyrosine Hydroxylase and Protein Assay. For measurement of tyrosine hydroxylase activity, five cultured (48 hr) and five control SCG were homogenized individually in 100 μl of 5 mM Tris buffer (pH 6.0) containing 0.1% Triton X-100. A 10-μl aliquot was used for measurement of enzyme activity as described previously (13), except that a saturating concentration of the cofactor 6-methyl-5,6,7,8-tetrahydropterine (3.2 mM) was used and the pH of the reaction mixture was 6.0. Ganglion protein was determined by the bichromonic acid (BCA) protein assay (Pierce).

RNA Isolation and Northern Blot Analysis of VIP/PHI mRNA. VIP/PHI mRNA was examined by Northern blot analysis. Total RNA was isolated from the ganglia by the guanidinium isothiocyanate–acid phenol method (14), separated in a denaturing 1.2% agarose gel, and blotted to nylon-linked nitrocellulose (GeneScreen; NEN Research Products). The integrity and relative amount of RNA loaded in each lane of the gel were assessed by staining the blot with methylene blue. RNA markers of known size (RNA ladder; Gibco/BRL) were used to estimate the sizes of RNA hybrids.

A 32P-labeled RNA probe was generated by transcription in the presence of [α-32P]UTP from a 380-base-pair fragment of the cDNA for rat VIP/PHI inserted into pSP64 (ref. 15; from J. S. Fink), using a kit from Promega. The specific activity of the probe generated was about 107 cpm/μg of RNA. The blot was prehybridized for 6 hr at 60°C and then hybridized overnight at 60°C in fresh prehybridization solution with the 32P-labeled RNA probe added. After hybridization, the blot was washed once at 60°C with 5x SSC (1x SSC = 0.15 M sodium chloride/0.015 M trisodium citrate) plus 0.1% SDS, twice at 65°C with 1x SSC plus 0.1% SDS, and once at 70°C with 0.2x SSC plus 0.1% SDS. The blots were exposed to X-Omat AR film (Kodak) together with an intensifying screen (Fisher) for 2–7 days at −80°C.

Statistics. The significance of differences was determined by a Student’s t test or a one-way analysis of variance, followed by post-hoc t tests with Bonferroni correction.

RESULTS

VIP-IR Increases in SCG in Organ Culture. Placing adult SCG in organ culture led to a 6-fold increase in their content of VIP-IR after 24 hr in culture and a 31-fold increase after 48 hr (Fig. 1). To compare the VIP-IR in the extract with authentic VIP, peptides were extracted from a ganglion cultured for 48 hr, separated by HPLC with gradient elution, and assayed by radioimmunoassay. Both the standard and the extract exhibited single peaks, with the same retention time, on HPLC (Fig. 2).

When both VIP- and NPY-IR were measured in individual ganglia that had been cultured for 24 hr, there was a significant increase in immunoreactivity for both peptides, although the change in VIP was considerably larger (7.3-fold vs. 2.5-fold; 24 hr vs. 0 hr; Table 1). In these same ganglia, there was no change in the total protein content (cultured, 130 ± 6; control, 129 ± 8 μg per ganglion). In addition, the activity of tyrosine hydroxylase, the enzyme that catalyzes the rate-limiting step in catecholamine biosynthesis, was measured to determine whether it changed under the same culture conditions. No increase in tyrosine hydroxylase activity was seen after 48 hr in organ culture (cultured, 56 ± 8; control, 53 ± 6 pmol of dopa per μg of protein per hr), as previously reported (8).

Cellular Location of the Increased VIP- and PHI-IR. When SCG cultured for 48 hr were examined by immunohistochemistry, a dramatic increase in the number of neuronal cell bodies and processes exhibiting VIP-IR was observed (Fig. 3 A and B). In addition, a comparable increase in immunostaining for PHI-IR was seen (Fig. 3 C and D). Whereas cells

![Fig. 1. Increases in VIP-IR in adult SCG maintained in organ culture. Ganglia were removed from rats and either frozen directly (t = 0) or placed in organ culture for 24 hr (t = 24) or 48 hr (t = 48). Data represent the means ± SEM of five SCG. *, P < 0.01 vs. t = 0; **, P < 0.005 vs. t = 0; *** P < 0.005 vs. t = 24.](image1)

![Fig. 2. Chromatography of VIP-IR in an explanted ganglion. After a SCG was maintained in culture for 48 hr, peptides were extracted with acid, the extract was subjected to HPLC, and the collected fractions were assayed by radioimmunoassay to determine whether the VIP-IR in the SCG (solid line) was coeluted with authentic VIP (dotted line).](image2)
Table 1. Changes in VIP- and NPY-IR in cultured ganglia

<table>
<thead>
<tr>
<th>Time in culture, hr</th>
<th>VIP-IR, pg per SCG</th>
<th>NPY-IR, ng per SCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.2 ± 1.3</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>24</td>
<td>46 ± 8.2*</td>
<td>15 ± 0.1**</td>
</tr>
</tbody>
</table>

Ganglia were either frozen immediately after removal from the animal (0 hr) or maintained in organ culture for 24 hr. Data represent the means ± SEM for five or six ganglia. * P < 0.05 vs. 0 hr; ** P < 0.001 vs. 0 hr.

showing VIP- or PHI-IR were present at a frequency of <1 cell per section in control ganglia (ref. 9 and this study), about 10 cells per section were found in cultured ganglia. These labeled neurons (and processes) were distributed throughout the ganglion, among a larger population of unlabeled neurons. When colocalization studies were performed both VIP- and PHI-IR were found in the same neurons (data not shown).

Possible Factors Involved in the Increase in VIP-IR. Since NGF was normally not added to the culture medium and since placing the ganglia in organ culture deprives them of target-derived NGF, the effect of adding NGF (100 ng/ml) to the culture medium was examined. NGF neither affected the magnitude of the increase in VIP-IR nor altered total ganglion protein (data not shown).

The increase in VIP-IR in organ culture could have been caused by the presence of a stimulatory factor(s) in the newborn calf serum added to the normal culture medium. To examine this possibility, ganglia were cultured in either serum-containing BGJb medium or a chemically defined medium (supplemented Ham's F12). A comparable increase in VIP-IR was seen under both conditions (content at 0 hr was 8 ± 1.6; at 24 hr with serum-containing medium, 52 ± 7.4; and at 24 hr with defined medium, 68 ± 13 pg per SCG). Peptide immunoreactivity also increased to a similar extent when ganglia were cultured in BGJb medium without serum (data not shown).

Biochemical Mechanism of the Increase in Peptide Immunoreactivities. The increase in VIP-IR that occurred when ganglia were placed in organ culture for 24 hr was completely blocked when the ganglia were cultured in the presence of either an inhibitor of transcription (actinomycin D, 1 µg/ml) or an inhibitor of translation (anisomycin, 100 µM) (Fig. 4A). Neither drug affected the total protein content of the ganglia. While anisomycin also completely blocked the 2.4-fold increase in NPY-IR, actinomycin D had only a partial (55%) blocking effect (Fig. 4B).

The blockade of the increase in VIP-IR by actinomycin D is consistent with the hypothesis that there is an increase in the level of VIP/PHI mRNA in cultured ganglia. This hypothesis was examined directly by Northern blot analysis of total RNA extracted from control and cultured ganglia. For this, an RNA probe complementary to rat VIP/PHI mRNA was used. The results indicate a large increase in the level of this mRNA in organ culture (Fig. 5). A hybridizing species of 1.7-kb RNA, corresponding to the size of VIP/PHI mRNA observed in previous studies (11–13), was not detected in extracts of single control ganglia. Such a species was, however, detectable in blots of pooled samples of three control ganglia, after a long (7-day) autoradiographic exposure, indicating that a small amount of VIP/PHI mRNA is normally present in the adult SCG. In RNA extracted from an individual ganglion that had been cultured for 24 hr, a 1.7-kb RNA was clearly detectable. In addition, a second, much less intense band could be seen at about 1 kb, as also observed previously (13) in RNA extracted from rat brain. After 48 hr in culture, both the major and minor transcripts were significantly more prominent than after 24 hr.

DISCUSSION

Increases in VIP-IR, PHI-IR, and VIP/PHI mRNA in Organ Culture. After SCG are placed in organ culture for 48 hr, their levels of VIP-IR and VIP mRNA and the number of neuronal cell bodies and nerve processes exhibiting VIP-IR
increase dramatically. In addition, PHI-IR increases in the same cell bodies and processes. VIP and PHI belong to the same peptide family, the secretin–glucagon family (16); have similar distributions (e.g., refs. 8, 9, and 17) and effects (e.g., refs. 18 and 19) in the nervous system; and are encoded by the same mRNA (20–22). Using an antisense RNA probe generated from a rat cDNA fragment that contains the entire coding regions for both VIP and PHI and 69 base pairs of the 3′ untranslated region (15), we could only barely detect this mRNA in control ganglia but found a large signal in ganglia after 48 hr in culture. Thus, the increases in both VIP- and PHI-IR in these sympathetic neurons are likely to be due, at least in part, to an increase in the level of their common mRNA rather than simply to increased accumulation of the peptide in the cell bodies of axotomized sympathetic neurons. The increase in VIP-IR (or PHI-IR) is not completely blocked by inhibitors of either RNA or protein synthesis, suggesting further that the increase in peptide level is not simply due to posttranscriptional and/or posttranslational changes. Why some, but not all, cultured neurons express VIP- or PHI-IR under the conditions examined remains to be determined.

As an index of the specificity of these peptide changes, we looked for changes in NPY-IR and found a small, though significant, increase in the content of this peptide in the SCG. It should be noted that NPY-IR, unlike VIP- and PHI-IR, is already found in a large number of postganglionic neurons prior to their placement in explant culture (23). The lack of increase in tyrosine hydroxylase activity and total ganglion protein also suggests that the changes in VIP- and PHI-IR do not represent a nonspecific increase in all ganglion proteins.

What Might Be the Stimulus for the Increase in VIP- and PHI-IR? When the SCG is placed in organ culture, its principal neurons are (i) decentralized, (ii) axotomized, and (iii) exposed to a somewhat different humoral environment than found in vivo. The importance of changes in the humoral milieu of the neurons in organ culture on the increases in VIP- and PHI-IR is difficult to assess. Stimulatory factors in the newborn calf serum that is included in the culture medium could be important; however, our finding that increases of similar magnitude occur in a chemically defined medium makes this unlikely. Another possibility is that certain inhibitory factors—such as specific hormones, differentiation factors, or growth factors (see below)—that are present in vivo may be missing (or at low concentration) in our culture medium.

We have previously shown (24) that decentralization increases VIP-IR in the SCG; however, this change differs in two ways from that reported here. First, the increase that occurs in vivo 48 hr after the SCG is decentralized is much smaller, ≈2-fold. Second, immunohistochemical studies have shown that the principal change is in the number of immunostained nerve processes rather than cell bodies, though it is difficult to rule out a doubling in immunostained cell bodies, given the infrequency of their occurrence.

Preliminary results, on the other hand, raise the possibility that cutting the axons of postganglionic sympathetic neurons plays an important role in the increase in VIP-IR. Thus, when the postganglionic internal carotid nerve of the SCG was cut, there was a large increase in VIP-IR detected by radioimmunoassay and in the number of immunostained nerve processes rather than cell bodies (25). Interestingly, cutting the peripheral process of another cell type, the adult sensory neuron, has been shown to increase the neuron's content of VIP-IR and VIP/PHI mRNA, while decreasing its content of substance P-IR and preprotachykinin mRNA (26–28). These data raise the possibility that VIP-IR is negatively regulated in adult sympathetic (and perhaps sensory) neurons by a target-derived factor.

The level of one target-derived factor, NGF, is known to decrease dramatically in axotomized sympathetic neurons, since the primary source of NGF relevant to sympathetic neurons is their target tissues (20). Thus NGF might suppress VIP expression in sympathetic neurons. In fact, NGF had no effect on the increase in VIP-IR in the SCG. This contrasts with the ability of NGF to reverse several effects caused by axotomy of sympathetic neurons, in vivo, such as chromatolysis and decreased ganglionic transmission (21) and decreased tyrosine hydroxylase activity (22).

Studies on neonatal sympathetic neurons in cell culture have identified several factors that may be involved in the development of a VIPergic phenotype in vivo in sympathetic neurons that innervate foot pads. Leukemia inhibitory factor, which is present in heart cell-conditioned medium, and a factor recently isolated from rat foot pads induce the expression of VIP in neonatal sympathetic neurons in cell culture, in addition to stimulating their spreading. Moreover, a second factor in conditioned medium induces VIP expression without affecting acetylcholine synthesis (29). Finally, ciliary neurotrophic factor has been shown to increase both of these biochemical measures in cultured sympathetic neurons (42). If, however, any of these factors play a role in the stimulation of VIP-IR observed in the current study, it must be assumed that they are produced in sufficient quantity by cells in the ganglion itself, when placed in organ culture.

Changes in the Level of Other Peptide Immunoreactivities in SCG in Organ Culture. As already noted, NPY-IR also increased in cultured ganglia. While the cause of this increase is unknown, we do know that decentralization does not account for the change, since we have found that NPY-IR in the SCG significantly decreases (though only by 30%) 1 week after decentralization (25). While we have not looked for changes in NPY mRNA, it is interesting that actinomycin D blocked only half of the increase in NPY-IR normally seen in cultured ganglia, suggesting a difference in the extent of involvement of gene transcription in the increase of NPY-IR compared with that of VIP-IR. Perhaps, since NPY-IR is already being synthesized by many postganglionic neurons in control ganglia, the level of this peptide is more sensitive than that of VIP to decreases in peptide transport out of the ganglion and to decreases in peptide release, both of which might be expected to occur in organ culture.

As already noted, neonatal and adult SCG placed in organ culture also increase their level of substance P-IR (7). While the increase in substance P-IR has been studied primarily in
neonatal ganglia, it appears to have some similarities with the increase in VIP-IR in adult ganglion. For example, in both cases there are increases in peptide content, in the number of immunostained neurons, and in the level of the corresponding mRNA (31). In addition, however, there are interesting differences between the two peptides. The increase in substance P seems to occur more slowly in cultures of adult ganglia than the increase in VIP-IR (7), and no increase in substance P occurs in the adult SCG in vivo after axotomy (32). In neonatal ganglion explants, actinomycin D only partially blocks the increase in substance P-IR (4, 33). Thus, while adult sympathetic neurons exhibit phenotypic plasticity with respect to expression of both VIP and substance P, it appears that there are differences in the cellular and molecular mechanisms involved in the regulation of the two peptides.

Possible Functional Implications of the Changes in VIP and PHI-IR. VIP produces a variety of effects on the rat SCG. For example, in adult neuronal cell bodies/dendrites, VIP increases levels of cAMP (19, 34) and inositol phosphate (35), increases the activity and extent of phosphorylation of tyrosine hydroxylase (19, 36), enhances synaptic transmission in the ganglion (37), and potentiates the electrophysiological response to muscarinic agonists (38). In those systems where PHI has also been examined, it produces effects similar to those of VIP, with the exception of the increase in inositol phosphate (35). Thus, if the changes in VIP- and PHI-IR seen in organ culture can also occur under certain conditions in vivo and if the synthesized peptides are released, they could act as autocrine and/or paracrine factors. Perhaps most intriguing is the possibility that VIP acts as a trophic factor for maintaining injured sympathetic neurons and/or promoting fiber outgrowth. Such a role has been proposed for VIP during normal embryonic development of sympathetic neurons (39) and retinal ganglion cells (40), based on effects of the peptide observed in cell culture. An indirect trophic role for VIP has been observed in cultures of spinal cord neurons, where the peptide can release a trophic factor from non-neuronal cells that promotes neuronal survival (41). Finally, since VIP-IR increases in sensory neurons when their peripheral process is cut (26), it will be interesting to determine whether a similar mechanism of regulation of VIP expression occurs in both of these neural crest-derived cell types and whether VIP plays a similar role(s) in both after axotomy.

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