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Neuropeptide Y-like immunoreactivity in rat cranial parasympathetic neurons: Coexistence with vasoactive intestinal peptide and choline acetyltransferase

(autonomic nervous system/peptide colocalization/norepinephrine/acetylcholine)

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ABSTRACT **Neuropeptide Y (NPY) is widely distributed in the sympathetic nervous system, where it is colocalized with norepinephrine. We report here that NPY-immunoreactive neurons are also abundant in three cranial parasympathetic ganglia, the otic, sphenopalatine, and ciliary, in the rat. High-performance liquid chromatographic analysis of the immunoreactive material present in the otic ganglion indicates that this material is very similar to porcine NPY and indistinguishable from the NPY-like immunoreactivity present in rat sympathetic neurons. These findings raise the possibility that NPY acts as a neuromodulator in the parasympathetic as well as the sympathetic nervous system. In contrast to what has been observed for sympathetic neurons, NPY-immunoreactive neurons in cranial parasympathetic ganglia do not contain detectable catecholamines or tyrosine hydroxylase (EC 1.14.16.2) immunoreactivity, and many do contain immunoreactivity for vasoactive intestinal peptide and/or choline acetyltransferase (EC 2.3.1.6). These findings suggest that there is no simple rule governing coexpression of NPY with norepinephrine, acetylcholine, or vasoactive intestinal peptide in autonomic neurons. Further, while functional studies have indicated that NPY exerts actions on the peripheral vasculature which are antagonistic to those of acetylcholine and vasoactive intestinal peptide, the present results raise the possibility that these three substances may have complementary effects on other target tissues.**

Neuropeptide Y (NPY) is structurally related to members of the pancreatic polypeptide family but appears to be localized exclusively in central and peripheral neurons (1, 2). In the periphery, NPY is present in sympathetic neurons that innervate the vasculature (3, 4), heart (5, 6), urogenital tract (3, 7), and iris (8). A number of biological actions have been reported for NPY, including a potent vasoconstrictor action on peripheral and cerebral vessels (3, 4, 9) and inhibition of synaptic transmission in the vas deferens, uterus, urinary bladder, and heart (3, 10, 11). NPY may therefore act as a neuromodulator in the sympathetic nervous system.

NPY-immunoreactive sympathetic neurons contain the catecholamine-synthetic enzymes tyrosine hydroxylase [TyrOHase; L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] and dopamine β-hydroxylase [3,4-dihydroxyphenethylamine,ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], indicating that NPY is colocalized with norepinephrine in these neurons (3, 12–14). In contrast, there is little overlap between populations of sympathetic neurons that contain NPY and those that contain vasoactive intestinal polypeptide (VIP) (12, 14). The minority population of sympathetic neurons that contain VIP has been found to lack catecholamine-synthetic enzymes; as is the case for VIP-containing parasympathetic neurons, there is evidence that at least some of the neurons in this population are cholinergic (12, 15, 16). The notion has emerged from these studies that coexpression of NPY with norepinephrine, and of VIP with acetylcholine, is an organizational principle in the autonomic nervous system (14).

To explore further the relationship between NPY expression and the expression of other neurotransmitter phenotypes in autonomic neurons, we have examined cranial parasympathetic neurons for the presence of NPY. The present work shows that NPY-immunoreactive neurons are abundant in rat cranial parasympathetic ganglia and examines the colocalization of NPY immunoreactivity with TyrOHase, VIP, and the cholinergic enzyme choline acetyltransferase (ChoAcTase; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) in these neurons.

MATERIALS AND METHODS

Cytochemistry. The NPY antiserum (Amersham) was raised in rabbit against synthetic porcine NPY. The VIP antiserum, a gift from P. Hogan (Harvard Medical School), was raised in rabbit against a carbodiimide conjugate of synthetic VIP (Boehringer Mannheim) to bovine serum albumin. The TyrOHase antiserum, a gift from J. Thibault, was raised in rabbit against TyrOHase purified from rat pheochromocytoma tumors (17). The ChoAcTase antiserum, a gift from F. Eckenstein, was raised in mouse against ChoAcTase purified from pig brain (18).

NPY, TyrOHase, and VIP immunoreactivities were examined with the indirect immunofluorescence method. Adult rats were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.3) for 10 min. Tissues were removed and incubated in the same fixative for 1 hr, rinsed in phosphate buffer, and equilibrated for 24 hr in 30% sucrose in phosphate buffer. Cryostat sections (6–10 μm) were cut and mounted on gelatin-coated slides. Slides were rinsed with phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.4) and incubated overnight at room temperature in humid chambers with antisera against NPY, TyrOHase, or VIP (at dilutions of 1:500, 1:1000, and 1:1000, respectively) in incubation buffer (0.01 M sodium phosphate, pH 7.3/0.5 M NaCl/0.2% Triton X-100/0.1% NaN_3/5% bovine serum albumin). The sections were then rinsed with

Abbreviations: NPY, neuropeptide Y; TyrOHase, tyrosine hydroxylase; VIP, vasoactive intestinal peptide; ChoAcTase, choline acetyltransferase.

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phosphate-buffered saline and incubated for 2 hr at room temperature with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Tago, Burlingame, CA) diluted 1:400 in incubation buffer. After rinsing, the sections were coverslipped in glycerol/ethanol (1:1) and examined by epifluorescence using a rhodamine filter set.

ChoAcTase immunoreactivity was examined using the peroxidase-antiperoxidase method. Sections were prepared as above, except that the fixative used was 4% paraformaldehyde containing 15% (vol/vol) saturated picric acid, and the tissues were postfixed for only 30 min. Sections were rinsed in Tris-buffered saline (150 mM NaCl/100 mM Tris·HCl, pH 7.4) and incubated overnight at room temperature in primary antibody diluted 1:1000 in incubation buffer (Tris-buffered saline containing 1% bovine serum albumin, 0.5% Triton X-100, 10% rabbit serum, and 0.1% NaN_3). The sections were rinsed several times and then incubated for 1 hr at room temperature in affinity-purified rabbit anti-mouse immunoglobulins (Boehringer Mannheim) diluted 1:40 in incubation buffer containing 10% rat serum. After rinsing, the sections were incubated for 90 min at room temperature in a mouse monoclonal peroxidase-antiperoxidase complex (Sternberger-Meyer, Jarrettsville, MD) diluted 1:80 in incubation buffer containing 10% rat serum. The sections were then cycled a second time through the rabbit anti-mouse antiserum and mouse peroxidase-antiperoxidase. The sections were treated with 3,3'-diaminobenzidine (1 mg/ml; Sigma) and 0.01% hydrogen peroxide in 0.1 M Tris·HCl (pH 7.2), dehydrated, and mounted in Permount (Fisher).

No specific staining was observed when normal rabbit or mouse serum was substituted for the TyrOHase or ChoAcTase antiserum, respectively. Staining with the VIP antiserum was abolished by preincubating the antiserum with 10 μM synthetic VIP before applying it to the sections. Preabsorption controls carried out with the NPY antiserum are presented in *Results*.

Catecholamines were visualized in fresh-frozen tissue sections by the glyoxylic acid fixation method (19).

Radioimmunoassay. Frozen tissues were boiled in 0.5 M acetic acid for 5 min, homogenized, and boiled for another 5 min. The homogenates were spun for 10 min in a Beckman model B Microfuge, and the supernatants were removed and evaporated in a Savant Speedvac vacuum concentrator. Sample material or unlabeled NPY (Bachem Fine Chemicals, Torrance, CA) standards in duplicate were incubated overnight at 4°C in polystyrene tubes with NPY antiserum (Amersham) and 3000 cpm of ^{125}I-labeled NPY (Amersham) in assay buffer (50 mM sodium phosphate, pH 7.4/10 mM EDTA/0.1% bovine serum albumin/20 mM NaN_3) in a final volume of 250 μl. One hundred microliters of a suspension containing 2.5 mg of charcoal (Norit SG, from MCB Chemical, Norwood, OH), 0.6 mg of dextran, and 6.25 μl of fetal bovine serum in 10 mM sodium phosphate buffer (pH 7.5) was added to each tube, and the tubes were centrifuged at top speed in a Sorvall GLC centrifuge for 15 min. The supernatant and pellet were separated and the radioactivity in each was measured with a gamma counter to determine percent binding. The detection limit of the assay was 40 fmol of NPY.

High-Performance Liquid Chromatography. NPY-like immunoreactivity was partially purified from acetic acid extracts on a C_{18} Sep-Pak column (Waters Associates) and fractionated by reverse-phase HPLC employing a C_{18} column (μBondapak, 3.9 × 300 mm, Waters Associates), a flow rate of 0.5 ml/min, and a linear gradient of acetonitrile. Nonhydrophobic substances were eluted isocratically in 20% acetonitrile/0.1% trifluoroacetic acid for 10 min before beginning the gradient, which ascended to 65% acetonitrile/0.1% trifluoroacetic acid over 30 min. Fractions (0.5 ml) were collected and evaporated for radioimmunoassay.

RESULTS

Three cranial parasympathetic ganglia, the otic, ciliary, and sphenopalatine, were examined for the presence of NPY-like immunoreactivity in the rat. NPY-immunoreactive neurons were present in all three ganglia (Fig. 1). They were most abundant in the otic ganglion, where they comprised 60–80% of the total ganglion cell population; NPY immunoreactivity was present in approximately 15% and 30% of the neurons in the sphenopalatine and ciliary ganglia, respectively. NPY immunoreactivity was also seen in small intensely fluorescent cells in the sphenopalatine ganglion. Immunostaining in all three ganglia was eliminated when the NPY antiserum was preabsorbed with 1 μM synthetic porcine NPY but was unaffected by 5-fold higher concentrations of two structurally homologous peptides, peptide YY and neurotensin, or of the structurally unrelated peptide VIP.

We were interested in further characterizing the NPY-like immunoreactivity in parasympathetic neurons and, in particular, assessing its similarity to the NPY-like immunoreactivity present in sympathetic neurons. We therefore compared the reverse-phase HPLC elution profiles of NPY immunoreactivity extracted from the rat otic ganglion and the superior cervical ganglion, a sympathetic ganglion. (The otic ganglion was chosen for this purpose because it contained the highest concentration of NPY-immunoreactive neurons and, compared to the ciliary ganglion, is relatively large.) We found that the immunoreactive material extracted from the otic ganglion was eluted as a single narrow peak at approx-

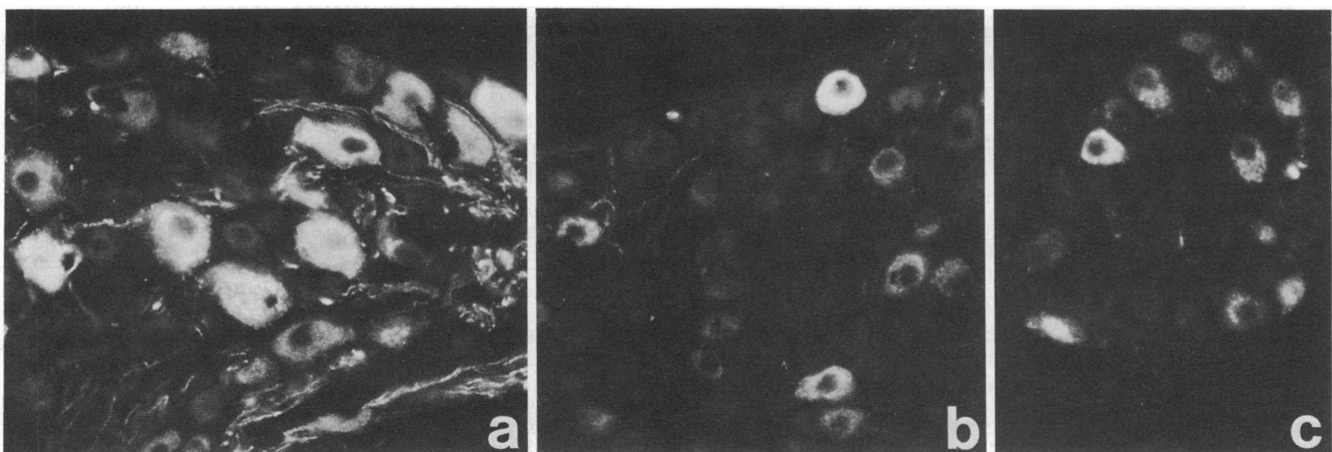

FIG. 1. Immunofluorescence micrographs of the otic (*a*), sphenopalatine (*b*), and ciliary (*c*) ganglia stained with antiserum to NPY. (×270.)

imately 45% acetonitrile (Fig. 2, upper profile). Its retention time was identical to that of NPY-like immunoreactivity extracted from a sympathetic ganglion, the superior cervical ganglion (Fig. 2, lower profile), consistent with the idea that sympathetic and parasympathetic neurons contain the same molecular form of NPY-like immunoreactivity. The immunoreactivity in both extracts was eluted in a position similar, but not identical, to that of synthetic porcine NPY. NPY has not yet been purified from rat tissue, and there may be a slight difference in the amino acid sequences of rat and porcine NPY.

No catecholamine histofluorescence was detected in principal neurons of the otic, sphenopalatine, or ciliary ganglia after treatment with glyoxylic acid. Subpopulations of cranial parasympathetic neurons have been found to contain immunoreactivity for the adrenergic enzyme TyrOHase (20–22); however, we found no correlation between the presence of TyrOHase and NPY immunoreactivities in neurons of the otic, sphenopalatine, or ciliary ganglia. TyrOHase immunoreactivity was generally exhibited by less than 1% of the neurons in the otic and sphenopalatine ganglia, suggesting that the vast majority of NPY-immunoreactive neurons in these two ganglia do not contain detectable TyrOHase immunoreactivity. This presumption was confirmed by analysis of serial sections stained for TyrOHase and NPY (Fig. 3). In the ciliary ganglion (not shown), which contains a relatively high (at least 30%) proportion of TyrOHase-immunoreactive neurons, the majority of NPY-immunoreactive neurons also contained TyrOHase immunoreactivity; however, a substantial fraction did not.

In agreement with previous reports (23, 24), we found that the majority of neurons in the sphenopalatine ganglion contained VIP immunoreactivity. VIP immunoreactivity was also present in most of the neurons in the otic ganglion, consistent with the reported presence of a plexus of VIP-immunoreactive fibers in the parotid gland (25), the primary target of the otic ganglion. Analysis of adjacent sections of the sphenopalatine and otic ganglia stained with antisera to VIP and NPY indicated that virtually all NPY-immunoreactive neurons in these two ganglia also contained VIP immunoreactivity (Fig. 4). This was not true, however, of NPY-immunoreactive neurons in the ciliary ganglion, since VIP

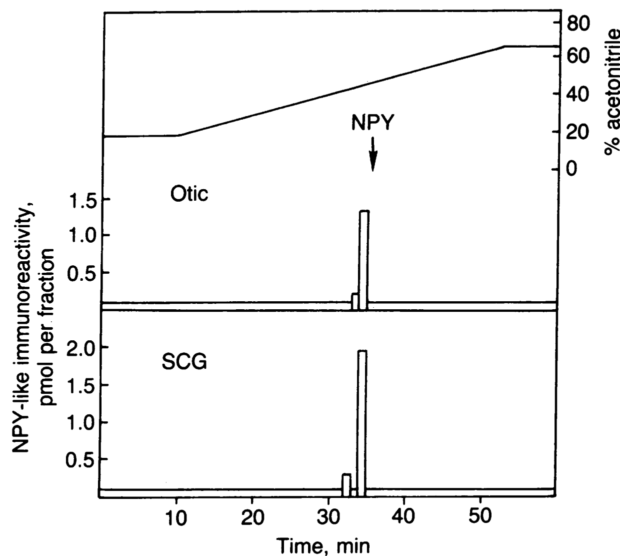

FIG. 2. HPLC elution profiles of NPY-like immunoreactivity in extracts of the otic ganglion and superior cervical ganglion (SCG). Values are expressed as molar equivalents relative to porcine NPY. The arrow marks the position of synthetic porcine NPY. The solid horizontal lines indicate the detection limit of the radioimmunoassay.

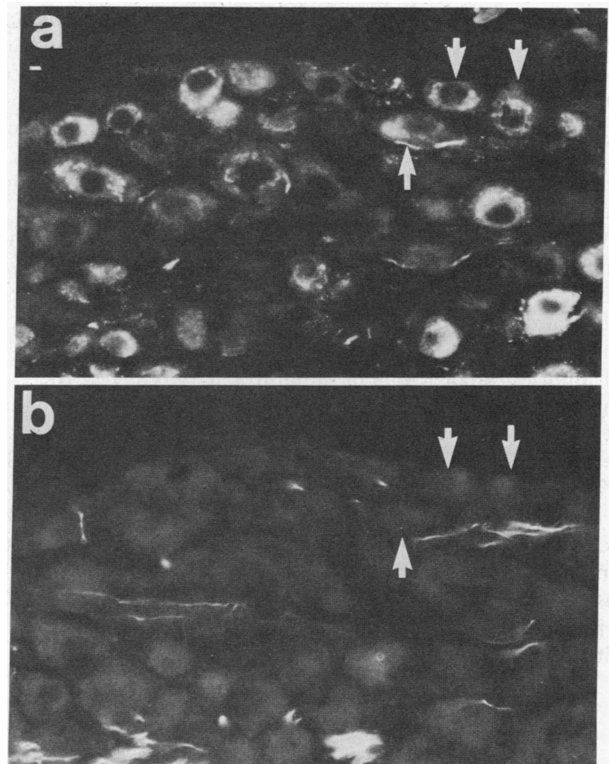

FIG. 3. Immunofluorescence micrographs of adjacent sections of the sphenopalatine ganglion stained with antiserum to NPY (*a*) or TyrOHase (*b*). No TyrOHase-immunoreactive cell bodies are present in *b*, although several TyrOHase-immunoreactive nerve fibers can be observed. Arrows indicate three cell bodies that stain for NPY but not TyrOHase. (×300.)

immunoreactivity was detectable in only occasional ciliary neurons.

It has generally been assumed that parasympathetic neurons are uniformly cholinergic. However, evidence for cholinergic function in parasympathetic neurons is based on physiological and biochemical studies that dealt with populations of neurons rather than individual neurons; there is no direct evidence that all parasympathetic neurons contain acetylcholine. To determine whether NPY coexists with acetylcholine in cranial parasympathetic neurons, we stained

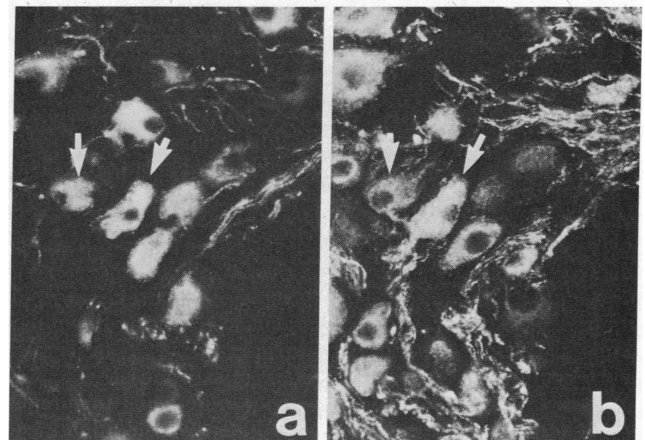

FIG. 4. Immunofluorescence micrographs of adjacent sections of the otic ganglion stained with antiserum to NPY (*a*) or VIP (*b*). Arrows indicate neurons that are stained with both the NPY and VIP antisera. (×270.)

adjacent sections of the otic, sphenopalatine, and ciliary ganglia either with NPY antiserum or with an antiserum to ChoAcTase, the enzyme that synthesizes acetylcholine. We found that all of the neurons in both the ciliary and otic ganglia contained ChoAcTase immunoreactivity, and serial-section analysis demonstrated that, as expected, all NPY-immunoreactive neurons also contained ChoAcTase immunoreactivity (Fig. 5 *b* and *c*).

Many NPY-immunoreactive neurons in the sphenopalatine ganglion also contained ChoAcTase immunoreactivity. However, whereas neurons in the otic and ciliary ganglia appeared quite homogeneous in their levels of ChoAcTase immunoreactivity, sphenopalatine neurons exhibited considerable variability in their levels of immunoreactivity (Fig. 5*a*). The majority of neurons in the ganglion contained low to moderate ChoAcTase immunoreactivity, and a small percentage were strongly immunoreactive. However, approximately 25% of the neurons were either very faintly immunoreactive or indistinguishable from background. While it is difficult to ascertain unambiguously whether any sphenopalatine neurons actually lack ChoAcTase, these findings raise the possibility that some sphenopalatine neurons may be purely peptidergic, or contain another small-molecule neurotransmitter yet to be identified.

DISCUSSION

We have shown that large subpopulations of neurons in three rat cranial parasympathetic ganglia, the otic, sphenopalatine, and ciliary, contain NPY-like immunoreactivity. Immunostaining in all three ganglia is eliminated when the NPY antiserum is preabsorbed with low concentrations of synthetic porcine NPY. The NPY-like immunoreactivity extracted from rat otic ganglia has an HPLC elution profile similar to that of synthetic porcine NPY and identical to that of NPY-like immunoreactivity extracted from the rat superior cervical ganglion, a sympathetic ganglion. These results indicate that NPY is present in parasympathetic neurons and suggest that NPY acts as a neuromodulator in the parasympathetic as well as the sympathetic nervous system.

Efforts have been made to correlate the peptide content of various neuronal populations with the presence of particular classical neurotransmitters or other neuropeptides. In the sympathetic nervous system, NPY has been found to occur in neurons that contain norepinephrine (3, 12–14) but not in those which contain VIP (12, 14). These findings raise the possibility that NPY expression in sympathetic neurons is regulated coordinately with that of norepinephrine and VIP. For instance, it seems possible that (*i*) NPY expression is induced coordinately with adrenergic traits or (*ii*) the induc-

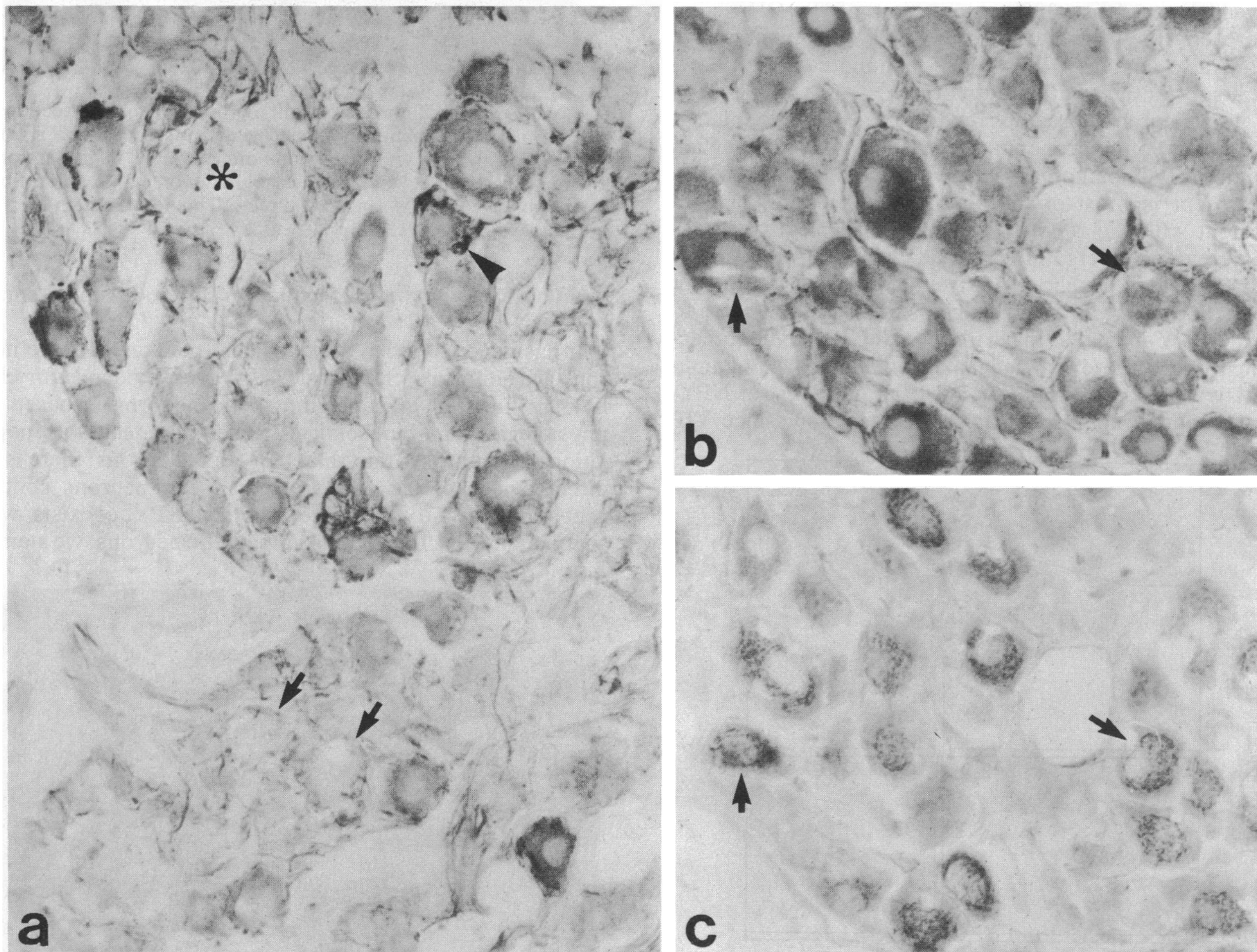

FIG. 5. Bright-field micrographs of the sphenopalatine ganglion stained with antiserum to ChoAcTase (*a*) and adjacent sections of the otic ganglion stained with antiserum to ChoAcTase (*b*) or NPY (*c*). (*a*) Preganglionic fibers, such as the one indicated by the arrowhead, are very darkly stained with the ChoAcTase antiserum, but there is considerable variation in the degree to which cell bodies in the sphenopalatine are stained. The asterisk marks a blood vessel, which is surrounded by small intensely fluorescent cells; these do not stain for ChoAcTase and therefore indicate the level of background staining. Arrows indicate two principal neurons whose degree of staining is not perceptibly different from background. (×340.) (*b* and *c*) Arrows indicate neurons in the otic ganglion that are stained with both the ChoAcTase and NPY antisera. (×340.)

tion of NPY expression involves a suppression of the ability to express VIP.

Our results indicate that the correlations observed in sympathetic neurons for the distribution of NPY relative to norepinephrine and VIP do not extend to other classes of neurons, not even those derived from the neural crest. First, we have shown that NPY immunoreactivity is present in cranial parasympathetic neurons, none of which contain detectable catecholamines. NPY immunoreactivity has also been found to occur in two other populations of autonomic neurons that lack catecholamines: enteric neurons (26) and intrinsic parasympathetic neurons of the heart (6, 27). Further, although NPY-immunoreactive cardiac neurons were reported to contain low levels of TyrOHase and dopamine β-hydroxylase immunoreactivity (6), we found no correlation between the presence of NPY and TyrOHase immunoreactivities in cranial parasympathetic neurons. Rather, we found that most NPY-immunoreactive cranial parasympathetic neurons contain immunoreactivity for ChoAcTase, consistent with the idea that they are cholinergic. Colocalization of NPY and ChoAcTase immunoreactivities was also reported to occur in guinea pig enteric neurons (26). Finally, we have found that in many cranial parasympathetic neurons, NPY coexists with VIP immunoreactivity. Thus, it appears that there is no strict rule governing the coexpression of NPY with other neurotransmitters in the autonomic nervous system: NPY can, but does not necessarily, occur together with norepinephrine, VIP, or acetylcholine. These results suggest that any mechanisms which exist to coregulate NPY expression with that of other neurotransmitters operate differently in the sympathetic and parasympathetic nervous systems.

The codistribution of NPY with norepinephrine in the sympathetic nervous system is consistent with functional studies that have shown that NPY is a potent vasconstrictor (3, 4, 9). The functional consequences of the colocalization of NPY with VIP and acetylcholine in cranial parasympathetic neurons remain to be determined. Since both VIP and acetylcholine are vasodilators (28, 29), one would not expect NPY to be coreleased with them at vascular neuroeffector junctions. The present results therefore raise the possibility of physiological actions for NPY that are complementary rather than antagonistic to those of VIP and acetylcholine.

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