Colocalization of Somatostatin with GABA or Glutamate in Distinct Afferent Terminals Presynaptic to the Mauthner Cell

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The presence of somatostatin in afferent fibers impinging on the goldfish Mauthner (M-) cell was determined using immunohistochemical methods, combined with confocal and electron microscopy, and the relationship of this peptide with inhibitory and excitatory terminals was studied. Somatostatin-reactive boutons were present only on the distal part of the M-cell's lateral dendrite. Somatostatin immunoreactivity was observed in typical large myelinated club endings (LMCEs) corresponding to mixed (electrical and chemical) eighth nerve primary afferent fibers. The axoplasm of these fibers contained dense-core vesicles (DCVs) dispersed among round vesicles. We have made a novel finding that the excitatory transmitter glutamate is present in LMCEs. Colocalization of this amino acid with somatostatin was detected in 75% of these endings using postembedding staining with gold particles of various sizes. The other structures labeled by somatostatin antibody were found to be small vesicle boutons (SVBs), which establish symmetrical synapses and contain a population of pleiomorphic vesicles with DCVs scattered among them. Double labeling with antibodies against glutamic acid decarboxylase and GABA allowed the definition of three types of biochemically characterized terminals: [somatostatin-GABA], [GABA], and [somatostatin]. However, the occurrence of DCVs in SVBs stained for GABA alone suggests that neuropeptides other than somatostatin may also coexist with GABA in this class of boutons. The coexistence of somatostatin with both inhibitory and excitatory neurotransmitters acting on the same region of a postsynaptic cell is discussed in relation to the role postulated for this peptide in synaptic plasticity.

[Key words: somatostatin, GABA, glutamate, colocalization, dendrite, Mauthner cell, immunocytochemistry]

Various peptides are found in terminals of central neurons, where they are often stored in dense-core vesicles (DCVs) and colocalized with more classical neurotransmitters (Hökfelt, 1991). For example, it has been reported that GABA coexists with cholecystokinin, vasoactive intestinal peptide, neuropeptide Y, substance P, or somatostatin in the neocortex of the rat (Jones and Hendry, 1986). In the course of an ultrastructural study of

GABAergic innervation of the goldfish Mauthner cell, we observed the presence of numerous DCVs in small vesicle boutons (SVBs) as reported previously (Tuttle et al., 1987) and preferentially SVBs containing GABA. In an attempt to identify the related peptide, we focused our attention on somatostatin-14 for the following reasons. It is expressed in most representatives of the phylogeny (Argos et al., 1983), including fish (Buchanan et al., 1987; Christenson et al., 1991; Zupanc et al., 1991) in which a molecule identical to the mammalian somatostatin-14 has been identified (Epelbaum, 1986). Further, this cyclic neuropeptide may coexist with GABA in the lamprey spinal cord (Christenson et al., 1991).

The extremity of the M-cell lateral dendrite is richly innervated by glutamic acid decarboxylase (GAD)-immunoreactive boutons (Petrov et al., 1991) and the corresponding GABAergic boutons are adjacent to the large myelinated club endings (LMCEs) that comprise the main electrical and chemical excitatory inputs to this neuron (Furshpan, 1964; Korn and Faber, 1976; Faber and Korn, 1978), a spatial relationship between excitatory and inhibitory afferents comparable to that for distal dendrites and dendritic spines of hippocampal (Somogyi et al., 1984) and neostriatal (Takagi et al., 1983) neurons. In this article, we show that some of these inhibitory afferents contain both somatostatin and GABA. Surprisingly, we have also observed for the first time somatostatin immunoreactivity in excitatory terminals, that is, in LMCEs. Previous work has shown that long-term potentiation (LTP) occurs at these excitatory terminals (Yang et al., 1990) and that it involves activation of NMDA receptors. Further, the excitatory amino acid antagonist γ -D-glutamylglycine abolishes the EPSPs at this synapse (Woloszyn and Faber, 1988). Taken together, these results suggest that the transmitter for the chemical component of this mixed synapse is glutamate and, indeed, with postembedding immunocytochemistry we demonstrate now that glutamate is present in all the LMCEs where it is colocalized with the neuropeptide somatostatin-14 in about 75% of the endings studied.

Materials and Methods

Fifteen adult goldfish (*Carassius auratus*), 10–12 cm in length, were perfused through the heart with aldehyde fixative after being anesthetized with 0.30% 3-aminobenzoic acid ethyl ester (MS222; Sandoz) and paralyzed with Flaxedil (Rhône Poulenc; 1 μ g/mg body weight).

Fixation procedures. For fluorescence microscopy, animals were perfused for 20 min with fresh cold paraformaldehyde (4%) in 120 mM phosphate buffer (PB) at pH 7.4. After dissection, the brains were immersed overnight in paraformaldehyde and sucrose (40%) in phosphate-buffered saline (PBS), at 4°C. Brainstems were cut (30 μ m) with a freezing microtome and slices were treated for 30 min with 0.25% ammonium chloride in PBS to block the free aldehyde groups. For peroxidase ex-

Received Mar. 4, 1993; revised July 7, 1993; accepted July 15, 1993.

We thank Dr Streit (University of Zürich) for providing the monoclonal antibody, D. S. Faber for critical reading of the manuscript, and P. Caramelle for technical assistance and photography. C.S. is a recipient of a fellowship from the Ministère de la Recherche et de la Technologie. This work was supported by a grant (90-069) from DRET.

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periments, fish were treated as described above, except that a perfusion mixture of 2% paraformaldehyde and 2% glutaraldehyde was used in order to preserve somatostatin reactivity and the brain slices (30 μ m) were cut with a vibratome without sucrose treatment. For electron microscopy, four fish were perfused with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in PB at pH 7.4. This fixative allows visualization of GABA, glutamate, and somatostatin immunoreactivities. The brains were sectioned with a vibratome and the M-cell-containing slices (80 μ m) were kept in PBS.

Immunohistochemistry. For light microscopy, sheep anti-GAD and rabbit anti-somatostatin-14 (Amersham) antibodies were diluted in PBS at 1:2000 and 1:500, respectively. The floating sections were incubated with somatostatin antiserum or a mixture of the two antibodies overnight, at room temperature, and in presence of 0.25% Triton X-100 and 0.12% gelatin for double labeling experiments. After extensive washing with PBS, GAD and somatostatin were revealed with a donkey anti-goat coupled to tetramethyl isothiocyanate (TRITC; Nordic Immunological Labs) (1:200 in PBS) and a swine anti-rabbit coupled to fluorescein isothiocyanate (FITC; Nordic Immunological Labs) (1:200 in PBS), respectively, for 2 hr. The sections were finally mounted with Mowiol (Hoechst, Frankfurt, Germany) and examined either with a Zeiss microscope equipped for fluorescence or a confocal microscope (Molecular Dynamics, Sunnyvale, CA) coupled to a graphic station. Briefly, 15 successive optical sections (focus step size, 0.5 μ m) were obtained with appropriate settings of the argon laser wavelengths for excitation (488 and 514 nm) and long-pass filters (515 and 530 nm) for FITC and TRITC, respectively. Data from the same histological sample were collected consecutively at the identical depth for GAD and somatostatin, and were stored in a host computer. The background noise was reduced and the contrast was enhanced using a Gaussian ($3 \times 3 \times 3$) filter. Final images were reconstructed by superposing the two sets of digitized optical sections.

For peroxidase staining, slices were incubated overnight in the primary anti-somatostatin-14 polyclonal serum (1:500), for further assessment with an avidin-biotin-peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA). For this purpose, the floating sections were dipped sequentially for (1) 2 hr in a secondary biotinylated antibody (donkey anti-rabbit, 1:200; Amersham) and (2) 2 hr in ABC (1:100). The bound horseradish peroxidase (HRP) was visualized by using diaminobenzidine tetrahydrochloride (DAB) as the chromogen, by incubating preparation for 15 min in 0.03% DAB in 0.1 M Tris-HCl, pH 7.4, and then adding 0.03% H_2O_2 to the solution. The reaction was controlled visually and stopped by extensive washing with Tris-HCl. Sections were then dehydrated in graded alcohol, in order to enhance the contrast, and mounted on slide with Eukitt.

Postembedding technique. Vibratome sections (80 μ m) were incubated in osmium tetroxide (2%, 20 min), dehydrated in graded ethanol and embedded flat in Araldite. In one experiment, osmium tetroxide treatment was omitted and the dehydrated preparation was reacted with 1% ethanol phosphotungstic acid (EPTA) for 2 hr at 60°C. Serial ultrathin (silver to pale yellow) or semithin (0.5 μ m) sections were recovered on Formvar-coated nickel grids and treated as follows: (1) 1% H_2IO_6 in H_2O (7 min); (2) somatostatin-14 (1:500) or GABA (1:150 to 1:250) or glutamate (1:5000) antiserum in 0.05 M Tris buffer (pH 7.4) containing 0.9% NaCl, 0.1% Triton X-100 overnight at room temperature; (3) 0.5% polyethylene glycol (10 min) in Tris NaCl buffer; (4) goat anti-rabbit antibody coupled to 15 nm (Amersham) or 5 nm (Biocell) gold particles or goat anti-mouse antibody coupled to 5 nm gold particles (Amersham) diluted 1:50 in the buffer used in the previous step for 2 hr; (5) 2% uranyl acetate (15 min). Some serial sections were processed only with 2% uranyl acetate (15 min) followed by 1% lead citrate (2–5 min) in order to better visualize ultrastructure. For colocalization studies of GABA and somatostatin, alternate sections were reacted with each antibody. The coexistence of glutamate and somatostatin was revealed on the same section with gold beads of different sizes, using an anti-glutamate mouse monoclonal antibody and an anti-somatostatin rabbit polyclonal antiserum.

Antibodies used and their specificity. GAD polyclonal and glutamate monoclonal antisera, mAb 2D7 (kindly provided to us by Dr. Streit, University of Zürich), used in this study, were previously characterized (Oertel et al., 1984; Liu et al., 1989). The commercially available antiserum to somatostatin-14 (Amersham) was raised in rabbits using a synthetic peptide conjugated to human serum albumin and glutaraldehyde as the coupling agent. The particular cyclic structure (Epelbaum, 1986) of this peptide makes cross-reactivity of the antibody with other

neuropeptides unlikely. According to the manufacturer, no peptide other than somatostatin was recognized by these immunoglobulins. Since the staining we observed in fish brains matches the distribution of somatostatin in rat (see Discussion), the term “somatostatin immunoreactivity” is used in this article instead of the more precise designation of somatostatin-like immunoreactivity.

Control experiments. For light and electron microscopy, omission of the first antibody gave no staining ($n = 7$) and, in the case of double labeling experiments, no cross-reactivity ($n = 4$).

Analysis of immunostaining. Measurements of gold particle densities were made with a graphic tablet (Jandel Scientific) coupled with a microcomputer, and the results were analyzed with STATGRAPHICS software. In the case of LMCEs, we arbitrarily counted gold particles inside the area included between the point where these terminals lose their myelinated sheath and the presynaptic membrane. In the histograms, results from three animals, processed with the same immunohistochemical procedure, were pooled.

Results

Distribution of somatostatin-containing inputs

The single staining experiments with somatostatin-14 antiserum showed that somatostatin-containing afferents are segregated to a restricted region of the M-cell (Fig. 1). No immunoreactivity for the peptide was detected on the soma, including the axon cap (Fig. 1A), a specialized neuropil that surrounds the axon initial segment. At this brainstem level, a few varicose fibers (not shown), with a discontinuous staining, were observed close to the small ventral dendrites that originate from the soma, but never in direct contact with them. Numerous positive boutons were observed beyond the bifurcation of the lateral dendrite, with both fluorescent (not shown) and peroxidase (Fig. 1B) staining. The latter technique, which allows a glutaraldehyde fixation, gave a more intense labeling and therefore better visualization of immunoreactive boutons. The proportion of stained profiles was already decreased at the level of the bifurcation of the dendrite.

Ultrastructure of somatostatin-immunoreactive boutons

Postembedding immunogold labeling for somatostatin was used to determine the fine morphological features of stained terminals. This method, which is quite sensitive to the antigens, indicated that there is a low amount of somatostatin in individual terminals, and in order to differentiate labeled endings from unlabeled ones, the density of gold particles was computed in all SVBs and LMCEs. The background noise in the M-cell cytosol facing the selected boutons was determined with the same method. The raw data are shown as frequency histograms for the M-cell cytosol (Fig. 2A), SVBs (Fig. 2B), and LMCEs (Fig. 2C). These skewed histograms (Fig. 2B,C) indicate that all SVBs and LMCEs do not contain the same amount of somatostatin immunoreactivity. To differentiate the stained processes from the unstained ones, a threshold of 2.21 gold particles/ μm^2 was set. It corresponds to the average density plus SD (1.31 ± 0.90 gold particles/ μm^2 , $n = 62$) measured in the M-cell which is free from peptide. Profiles with a density above this limit were considered as labeled (Fig. 2D). The number of gold particles in the latter was 3.65 ± 1.89 ($n = 103$) in SVBs and 1.97 ± 0.84 ($n = 45$) in LMCEs after subtraction from density in the M-cell cytosol. The values in these endings are significantly different from the background noise (Student's t test, $p < 0.001$). On the distal dendrite, 65% of the SVBs (103 positive for 159 analyzed) and 75% of the LMCEs (45 positive for 60 analyzed) contained somatostatin immunoreactivity. The labeled SVBs had common ultrastructural features; namely, they (1) establish sym-

Figure 1. Preferential location of somatostatin labeled boutons in specific regions of the M-cell synaptic bed. *A*, Micrograph obtained with Nomarski optics showing the lack of immunoreactivity inside the axon cap, on the soma (*S*), and on the small ventral dendrites (arrowheads) issuing from it. *B*, Positively stained boutons (arrows) and presumed club endings (arrowheads) distal to the bifurcation of the lateral dendrite (*LD*). Scale bars: *A*, 30 μ m; *B*, 15 μ m.

metrical synaptic contacts with the target cell (Fig. 3*A*,*A*₂); (2) are filled with a pleiomorphic population of small vesicles, and large and numerous mitochondria usually situated away from the presynaptic membrane (Fig. 3*A*,*B*); and (3) also contain DCVs, which are better visualized in semithin sections stained with EPTA (Fig. 3*C*). Occasionally, accumulation of two or three 15 nm gold particles was observed over DCVs (see also Localization of somatostatin in nerve endings, below), but labeling was also always present over axoplasm and fewer particles were found over some mitochondria (Fig. 3*A*). These DCVs were not usually associated with the presynaptic active zone, and were observed anywhere in the bouton.

Colocalization of GAD, GABA, and somatostatin

The occurrence of symmetrical synapses and pleiomorphic vesicles in somatostatin-immunoreactive SVBs suggested that SVBs contain inhibitory neurotransmitters (ref. in Korn et al., 1989) such as GABA. To test for this possible colocalization we conducted double labeling experiments for GAD and somatostatin. As shown in Figure 4, peptide immunoreactivity is present in GAD-positive terminals (Fig. 4*A*,*A*₂). This colocalization is maximal on the distal part of the lateral dendrite whereas at its medial portion, only GAD staining was observed (not shown). The weak labeling, especially for somatostatin, led us to study regional distributions with confocal microscopy. Just proximal to the dendritic bifurcation, the majority of the boutons were only GAD immunoreactive and they were intermingled with a few [somatostatin-GAD]-positive terminals (Fig. 4*B*,*B*₂). At the most distal part of the dendrite we could distinguish three categories of afferents: [somatostatin-GAD], and [GAD] or [somatostatin] alone (Fig. 4*C*,*C*₂). The latter impinged exclusively on the extremity of the dendrite (Fig. 4*C*₂). All stained terminals had a mean diameter of $1.97 \pm 0.43 \mu$ m ($n = 22$), in agreement with previous electron microscopic data for the size of SVBs (Nakajima, 1974). Electron microscopic studies with GABA and somatostatin antisera on serial ultrathin sections confirmed the existence of these three populations of SVBs (Fig. 5). The [GABA-

somatostatin] population corresponds to SVBs that are enriched in DCVs (mean diameter $\pm$ SD = 109 ± 14 nm; $n = 40$) intermingled with pleiomorphic small vesicles (Fig. 5*A*,*A*₂), and carries symmetrical synapses (Fig. 5*A*,*A*₂). In this area, only few boutons had somatostatin immunoreactivity alone (Fig. 5*B*,*B*₂); they contained a mixed complement of small vesicles and of fewer DCVs. Some GABA-positive SVBs that did not contain somatostatin (Fig. 5*C*,*C*₂) displayed the same basic ultrastructural characteristics, including DCVs (104 ± 13 nm; $n = 43$), suggestive of another peptide.

Coexistence of glutamate and somatostatin

An unexpected finding with postembedding staining with the anti-somatostatin antiserum was the appearance of this peptide in the LMCEs (Fig. 6*A*). The ultrastructure of LMCEs was analyzed in serial ultrathin sections (Fig. 6*B*). In addition to gap junctions spanning the membrane apposed to the dendrite and attachment plate, there were asymmetrical synapses close to terminal borders with pre- and postsynaptic differentiation of the same length. The DCVs (105 ± 18 nm; $n = 30$) were usually observed associated with numerous round, small vesicles. Double staining experiments were conducted with anti-glutamate monoclonal and anti-somatostatin polyclonal antibodies in order to identify the excitatory neurotransmitter and its coexistence with somatostatin. This colocalization was observed in many LMCEs (Fig. 6*A*,*C*) that also displayed, as expected, DCVs (Fig. 6*A*) and glutamate immunoreactivity, a high density of gold particles being observed over mitochondria (Fig. 6*C*) and over small round vesicles. A quantitative analysis of the density of gold particles associated with glutamate immunoreactivity was conducted to ascertain that the concentration of glutamate was higher in these endings than in other terminals, particularly those containing GABA. The density measurements were performed as mentioned above for somatostatin. All afferent boutons (i.e., SVBs and LMCEs) impinging on the distal portion of the lateral dendrite were measured, since glutamate is used as a precursor of GABA or for metabolic purposes. As shown in

Figure 2. Distribution of particle densities indicative of somatostatin reactivity in different types of endings: histograms showing, in *A*, the control density in the M-cell cytosol apposed to positive terminals, and in *B* and *C*, the densities in stained SVBs and LMCEs, respectively. Corresponding means $\pm$ SD are 1.31 ± 0.9 gold particles/ μm^2 , $n = 62$, in *A*; 3.65 ± 1.89 gold particles/ μm^2 , $n = 103$, in *B*; and 1.97 ± 0.84 gold particles/ μm^2 , $n = 45$, in *C*. Correction for background in the M-cell was done for the means in *B* and *C*. The density over tissue-free resin was 1.1 ± 0.9 gold particles/ μm^2 , $n = 23$. *D*, Level of somatostatin immunoreactivity in SVBs and LMCEs. The values in SVBs (mean $\pm$ SEM, 4.96 ± 0.18 gold particles/ μm^2 ; $n = 103$) and in LMCEs (3.28 ± 0.12 gold particles/ μm^2 ; $n = 45$) are above that in M-cell cytosol (dashed line). Double asterisks indicate values significantly different ($p < 0.001$, Student's *t* test) from the M-cell cytosol value.

Figure 7, there was indeed a high density of gold particles in LMCEs (46.35 ± 13.11 gold particles/ μm^2 ; $n = 19$). Further, two populations of SVBs were distinguished on the basis of this criterion, namely, SVB1 and SVB2 with 13.26 ± 4.27 gold particles/ μm^2 ($n = 23$) and 30.17 ± 5.76 gold particles/ μm^2 ($n = 27$), respectively. These values were obtained after correcting for the M-cell cytosol density (9.86 ± 3.09 ; $n = 15$), and they are significantly different from each other (Student's *t* test; $p < 0.001$).

The detection of somatostatin in LMCEs with electron microscopy contrasts with the fact that confocal microscopy of immunofluorescent preparations did not provide evidence for the presence of this molecule in their parent axons. This discrepancy could be due to (1) the lower concentration of somatostatin in LMCEs as compared to SVBs, (2) the difficulty at the level of terminal endings to recognize them among other boutons with fluorescence, and finally (3) the use of paraformaldehyde for fluorescence. This aldehyde is not necessarily the best fixative for revealing binding sites of an antibody prepared against a glutaraldehyde coupled antigen (see Materials and Methods). Thus, we used an HRP method with glutaraldehyde fixation for a better correlation of light and electron microscopic observations. Under these conditions, a few LMCEs were somatostatin positive (Fig. 8*A₁*, *A₂*). At the extremity of the lateral dendrite, staining was seen in both *en face* (Fig. 8*A₁*; see also Fig. 1*B*) and transversal sections (Fig. 8*A₂*) of LMCEs surrounded by numerous negative profiles. Some large-diameter (5–16 μm) labeled fibers (Fig. 8*B,C*) were also found in the vestibular

nerve in agreement with the evenly distributed immunogold staining over club endings.

Localization of somatostatin in nerve endings

As shown above, postembedding immunocytochemistry with 15 nm gold particles showed somatostatin immunoreactivity in different SVBs and LMCEs axoplasm compartments (Figs. 3, 5, 6) and sometimes over DCVs, which are considered to be the site of the storage of peptides. However, since such large gold particles (15 nm) can reduce the accurate localization of the antigen, we performed another set of experiments, where the peptide was visualized with smaller (5 nm) gold particles (Fig. 9). With this marker, a preferential accumulation of gold particles over DCVs was generally observed in positive SVBs (Fig. 9*A,D*) and LMCEs (Fig. 9*B,C*) and was more conspicuous, given the larger number of (1) included particles, and (2) labeled DCVs. This result agrees with previous reports that peptides may be stored in DCVs (Takagi et al., 1983; De Biasi and Rustioni, 1988; Hökfelt, 1991). These stained terminals also displayed DCVs with fewer (Fig. 9*C*) or no (Fig. 9*A*) gold particles. Yet, as already noticed with 15 nm particles, we also observed the presence of 5 nm particles over vesicular compartments (Fig. 9*A*) and some mitochondria (Fig. 9*B*).

Somatostatin immunoreactivity in the vestibular nucleus

The observation of positive fibers in the eighth nerve, and the fact that the lateral dendrite of the M-cell lies in the vestibular nucleus, led us to analyze the distribution of somatostatin im-

Figure 3. Ultrastructural features of somatostatin-positive boutons revealed with gold particles on the distal part of the M-cell lateral dendrite. *A₁* and *A₂*, Ultrathin sections of the same ending showing an SVB (*A₁*) stained for somatostatin (densities: 5.41 gold particles/ μm^2 for SVB; 0.88 gold particle/ μm^2 in the M-cell cytosol facing the terminal) and the lack of gold particles in the absence of the first antibody (*A₂*). This SVB had DCVs (arrowheads) and symmetrical synapses (arrows). Note its location between two LMCEs (CE1, CE2). *B*, Two labeled terminals (T1, 4.7 gold particles/ μm^2 ; T2, 5.64 gold particles/ μm^2) with fewer DCVs (arrowheads) located near a negative bouton (asterisk; 1.5 gold particles/ μm^2). Here, the background noise estimated over a 19 μm^2 surface area is equivalent to 1.4 gold particles/ μm^2 . *C*, Semithin (0.5 μm) EPTA-treated section with numerous DCVs (arrowheads) and active zones (arrows) inside a somatostatin-positive terminal (9.54 gold particles/ μm^2 for SVB; 1.37 gold particles/ μm^2 in the M-cell cytosol). Scale bars: *A₁*, *A₂*, *B*, 1 μm ; *C* 0.5 μm .

Figure 4. Relative distribution of GAD (left column) and somatostatin (right column) in double-labeled afferent inputs. *A₁* and *A₂*, Presence of numerous GAD-positive boutons at the extremity of the dendrite (arrowheads); some are also stained for somatostatin (arrows); the pictures were obtained at the same focus. *B₁*–*C₂*, Confocal microscopic images of other preparations demonstrating that reactive profiles are positive for GAD, GAD-somatostatin, and somatostatin. Proximal to the bifurcation of the dendrite (*B₁*, *B₂*), an abundance of GAD-positive boutons (arrowheads) is in contrast with few double-stained terminals (arrows). Beyond the bifurcation (*C₁*, *C₂*), there are GAD-positive (arrowheads), and GAD-somatostatin-positive (arrows) profiles with a progressively increasing number of somatostatin-positive (crossed arrows) boutons. *A₁*–*C₁*, and *A₂*–*C₂*, TRITC and FITC filters for GAD and somatostatin, respectively. Scale bars: *A₁* and *A₂*, 10 μm ; *B₁*–*C₂*, 20 μm .

munoreactivity in this region. Immunoreactive profiles were observed in apposition to vestibular neurons located close to the tip of the M-cell lateral dendrite (not shown). However, numerous vestibular neurons did not receive detectable somatostatin input.

Double labeling experiments also revealed the presence of numerous GAD-positive boutons, in contact with vestibular neurons as well as double stained [somatostatin-GAD] profiles on a few of them (not shown). As mentioned above, the peroxidase reaction is more sensitive and it showed some somatostatin-positive vestibular neurons (not shown) lying close to unstained ones. In these cells, the somatostatin immunoreactivity was present in the whole cytoplasm without clear compartmentalization.

Discussion

In this study, we have found, in addition to the already reported (Somogyi et al., 1984; Jones and Hendry, 1986; Sloviter and Nilaver, 1987) coexistence of somatostatin with GABA, the presence of this peptide in glutamatergic excitatory endings. Several subtypes of afferents have been identified with respect to this and other transmitters, particularly at the extremity of the M-cell lateral dendrite, as summarized in Figure 10.

Specificity of the somatostatin-14 antiserum

Several lines of evidence support the notion that the somatostatin antiserum is as specific in fish as in other vertebrates. The pattern of staining, which includes neuronal cell bodies and varicose and thick fibers, corresponds to similar descriptions in rat (Finley et al., 1981; Takagi et al., 1983; Johansson et al., 1984; Sloviter and Nilaver, 1987), cat (Somogyi et al., 1984), and lamprey (Buchanan et al., 1987; Christenson et al., 1991). Furthermore, the restriction of the staining to the periphery of the brainstem, the reticular formation, its lack in the cerebellum (all not shown) and in the vestibular nucleus have been also reported in comparable areas of the rat using both immunocytochemistry (Finley et al., 1981; Takatsuki et al., 1981; Johansson et al., 1984) and biochemical measurements (Douglas and Palkovits, 1982). Furthermore, for a given structure that was labeled, others of the same type were negative, even though they had the same morphological features. Accordingly, gold particle densities in different profiles indicate that only a fraction of the SVBs (65%) and LMCEs (75%) are enriched in somatostatin immunoreactivity, in comparison with the M-cell background noise and with data from the tissue-free resin (1.1 ± 0.9 gold particles/ μm^2 ; $n = 23$). Both somatostatin-positive SVBs and LMCEs contained DCVs (105–109 nm) that exhibited accumulation of small gold particles. This is in agreement with reports that in the rat hypothalamic periventricular nucleus (Horvath et al., 1989) and neostriatum (Takagi et al., 1983) somatostatin immunoreactivity is present in large (100–160 nm) neurosecretory granules and in large DCVs (128 nm), respec-

tively. The observation of unstained DCVs in these positive terminals can be related to the presence of another peptide such as neuropeptide Y, which is known to coexist with somatostatin (Jones and Hendry, 1986), but may not share the same DCVs. Alternatively, this could be due to the postembedding staining used in this study, where only one side of the section was available for the reaction. On the other hand, in agreement with an earlier report of Takagi et al. (1983), we also found somatostatin immunoreactivity in axoplasm and associated with some mitochondria and with vesicular compartments in SVBs and LMCEs.

Segregation of somatostatin-positive boutons on the M-cell surface

Somatostatin was found to be present on the distal portion of the lateral dendrite in SVBs and LMCEs. The latter type of excitatory endings are only present in this area (Korn et al., 1989). Concerning the inhibitory SVBs, the restricted and stereotyped distribution of somatostatin terminals on the distal part of the lateral dendrite of the M-cell contrasts with the broader distribution of GABA and glycine. Although GABA-containing afferents are more numerous at the extremity of the lateral dendrite, they were found everywhere except in the axon cap (Petrov et al., 1991). Indeed, iontophoretic applications of GABA produce inhibitory responses in the M-cell (Faber and Korn, 1980) although glycinergic transmission is more widely distributed at the M-cell surface (ref. in Korn et al., 1989). The distribution of the somatostatin-containing fibers on the M-cell is similar to that in rat cortex, where they synapse with distal dendrites of pyramidal neurons (Takagi et al., 1983; Jones and Hendry, 1986; Sloviter and Nilaver, 1987), and in lamprey spinal cord, where the largest responses to somatostatin can be recorded on the dendritic shaft of the stretch receptor neuron (Christenson et al., 1991). This phylogenetic consistency suggests that somatostatin intervenes in the regulation of the dendritic properties. At the ultrastructural level, the majority of somatostatin-positive boutons contacting the M-cell correspond to SVBs (Nakajima, 1974).

The somatostatin-immunoreactive SVBs have structural features similar to terminals described by Tuttle et al. (1987), who recognized three types of SVBs, and more specifically, to their type A. Their small vesicles are more oval and round, and they contain more DCVs, mitochondria, and glycogen particles than type C. In the latter type, synaptic vesicles vary from flattened to round, whereas in SVB type B, most of them are flat.

Colocalization of GABA and somatostatin

The extremity of the M-cell lateral dendrite is innervated by GABAergic fibers (Petrov et al., 1991). Double labeling with GAD and somatostatin antisera revealed the coexistence of both markers in terminals distributed mainly beyond the bifurcation

Figure 5. Ultrastructural characteristics of the three populations of biochemically different SVBs revealed by postembedding staining for GABA (left) and somatostatin (right). *A₁* and *A₂*, Coexistence of GABA (dilution, 1:250) and somatostatin immunoreactivity (GABA, 12.63 gold particles/ μm^2 ; somatostatin, 8.95 gold particles/ μm^2) in the same bouton near a double-unstained terminal (asterisks). Note the widespread distribution of DCVs (arrowheads) and the presence of a synaptic contact (arrows) in the stained bouton. *B₁* and *B₂*, Example of a terminal positive for somatostatin (6.45 gold particles/ μm^2) but not for GABA (0.53 gold particles/ μm^2). This bouton bearing active zones (arrows) does not display any DCVs, in contrast to the nearby double-stained ending (asterisks; GABA, 9.20 gold particles/ μm^2 ; somatostatin, 6.54 gold particles/ μm^2). *C₁* and *C₂*, A GABA- (dilution, 1:150) labeled bouton (45.45 gold particles/ μm^2) that contained DCVs (arrowheads) and was not immunoreactive for somatostatin (0.60 gold particles/ μm^2). In all these pictures somatostatin background levels in the M-cell cytosol ranged from 1.3 to 2.1 gold particles/ μm^2 as measured from surfaces larger than $9 \mu\text{m}^2$. Scale bars, $0.5 \mu\text{m}$.

of the dendrite. Ultrastructural analysis of GABA and somatostatin with immunogold postembedding techniques revealed at least three populations of SVBs, a result that was also suspected by confocal microscopy analysis. The double-labeled SVBs, which represent one of these subpopulations, display numerous DCVs and mitochondria, therefore fulfilling some SVB type A features (see above). Since boutons containing GABA alone had the same characteristics, including DCVs, these organelles are not necessarily associated with somatostatin and they could be associated with other neuropeptides such as cholecystikinin, vasoactive intestinal peptide, or substance P as in the rat neocortex (reviewed in Jones and Hendry, 1986). The assumption that both [GABA-somatostatin] and [GABA] SVBs are of type A implies that their axons are unmyelinated and therefore probably originate from local interneurons (Tuttle et al., 1987). This notion is strengthened by observations in rat neostriatum, where all somatostatin-positive axons of medium-size aspiny neurons lack myelin (Takagi et al., 1983). The third, [somatostatin], population of SVBs has few DCVs and mitochondria, like SVBs of the C type; however, their scarcity did not allow for an accurate classification. This observation raises the problem of whether another fast-acting neurotransmitter is colocalized with somatostatin. Glycine would be a possible candidate since SVBs are generally inhibitory (ref. in Korn et al., 1989) and glycine receptors are present in this part of the cell (Seitanidou et al., 1988; Triller et al., 1990). In addition, somatostatin is associated with a high-affinity glycine uptake in the *Xenopus* retina (Smiley and Basinger, 1988).

Coexistence of glutamate and somatostatin

Glutamate immunoreactivity was found in LMCEs and in two groups of SVBs (1 and 2). The latter, which contain a pleiomorphic population of vesicles, are known to be inhibitory. The SVB1 are probably glycinergic boutons since the density of gold particles mainly found on mitochondria was only slightly above the background level. The SVB2 had a higher content in glutamate and DCVs were present in 85% of them. Hence, they correspond to GABAergic SVB type A (see above). The presence of glutamate in these endings accounts for its role as a substrate of GAD, the synthetic enzyme for GABA. The density of gold particles was the highest in LMCEs. These endings transmit both electrically and chemically (Furshpan, 1964; Korn and Faber, 1976; Lin and Faber, 1988) in agreement with electron microscopic (Roberston et al., 1963; Nakajima, 1974) and freeze-fracture (Tuttle et al., 1986) data. Previous iontophoretic (Diamond and Roper, 1973) and pharmacological (Wolszon and Faber, 1988) studies suggest that the chemical component of LMCEs is mediated by glutamate. This notion is strengthened by the demonstration of an NMDA-dependent LTP (Yang et al., 1990) and is confirmed here with immunocytochemistry. However, the presence of another excitatory amino acid such

Figure 7. Glutamate immunoreactivity in different terminal profiles contacting the distal part of the lateral dendrite. The values (mean number of gold particles/ $\mu\text{m}^2 \pm \text{SEM}$) are above that in the M-cell cytosol (dashed line; 9.86 ± 0.9 , $n = 15$). Note that the density of the labeling is the highest in LMCEs (56.21 ± 3.00 ; $n = 19$). Two populations of SVBs are distinct with respect to glutamate content: SVB1 (22.48 ± 1.04 ; $n = 24$) and SVB2 (40.03 ± 1.10 ; $n = 27$). Double asterisks indicate that values are significantly different when tested two by two ($p < 0.001$, Student's *t* test).

as aspartate can not be excluded. Indeed, the demonstration by Tracey et al. (1991) of the coexistence of both glutamate and aspartate in primary afferent neurons raises the possibility that these two neurotransmitters could be released simultaneously to activate NMDA receptors.

Although, somatostatin is commonly colocalized with inhibitory neurotransmitters, we have also found it in primary excitatory afferents. Coexistence of substance P, in primary glutamatergic fibers, was reported in the superficial laminae of rat spinal cord (De Biasi and Rustioni, 1988) and has been suggested for the adrenocorticotrophic hormone in the pigeon vestibular ganglion (Güntürkün and Deviche, 1992). In addition, the presence of DCVs in the LMCEs, away from the presynaptic grid, suggests that somatostatin could be released independently from glutamate at nonspecialized areas as suggested for another system (Zhu et al., 1986), and given this disposition, it could act on adjacent synapses as shown for glycine (Faber and Korn, 1988).

Functional implication

The coexistence of the same peptide in both excitatory and inhibitory endings raises the question of its function(s). In any case, the presence of somatostatinergic neurons in the vestibular nucleus, adjacent to the lateral dendrite, supports the hypothesis that these second-order interneurons synapse on the distal half of the M-cell lateral dendrite and that they probably are inhibitory. On the distal part of the M-cell lateral dendrite, somato-

Figure 6. Colocalization of glutamate and somatostatin immunoreactivity in an LMCE contacting the distal part of the lateral dendrite. *A*, Visualization of glutamate (5 nm gold particles) and somatostatin (15 nm gold particles) in the same club ending. The density of somatostatin labeling was 4.1 gold particles/ μm^2 in the terminal and 1.3 gold particles/ μm^2 in the M-cell cytosol (MC). That of glutamate was 70.67 gold particles/ μm^2 in LMCEs and 8.19 gold particles/ μm^2 in M-cell cytosol. Note the presence of a DCV (arrowhead) near the presynaptic membrane. *B*, Higher magnification of a nonlabeled adjacent section (area 1 in *A*) showing accumulation of small, round vesicles (crossed arrows) at the left edge of the ending, gap junctions (arrows), and chemical synapses (asterisks). Note the presence of a DCV (arrowhead). *C*, A detailed view of the same terminal (area 2 in *A*) obtained from a serial ultrathin section. Note the glutamate immunoreactivity over a mitochondrion (Mi) and in the vicinity of small vesicles (crossed arrows). Ap, attachment plate. Scale bars: *A*, 1 μm ; *B*, 0.5 μm ; *C*, 0.3 μm .

Figure 8. Eighth nerve inputs immunostained with somatostatin antiserum and their relationships with the distal lateral dendrite of the M-cell (DLD). *A₁* and *A₂*, Micrographs obtained from the same section, at different levels of focus with Nomarski optics. Tangential view (*A₁*, arrow) and transverse sections (*A₂*, arrows) of immunoreactive club endings close to unstained profiles (arrowheads). *B* and *C*, Somatostatin-containing afferents (arrows) within the eighth nerve (VIII N) and intermingled with negative profiles (arrowheads). The dashed line indicates the edge of the brainstem. Fixation with 4% paraformaldehyde for fluorescence (*B*) and 2% paraformaldehyde, 2% glutaraldehyde for peroxidasic reactions (*A*, *C*). Scale bars, 25 μ m.

statin released by these cells could act postsynaptically to complement GABA action by hyperpolarizing the M-cell through activation of a K^+ conductance as described in lamprey (Christenson et al., 1991). A presynaptic effect of somatostatin, which reduces GABA release, could also occur, as reported in the rabbit cerebral cortex at concentrations below those that elicit postsynaptic effects (Scharfman and Schwartzkroin, 1989). Such a disinhibition by somatostatin could also be produced by its release at the edges of excitatory afferents, thereby reinforcing excitation. This sequence could contribute to the induction of LTP on the lateral dendrite, by high-frequency stimulation that is known to increase somatostatin release (Bonanno et al., 1988) in other structures. Somatostatin could also potentiate electro-

tonic coupling via gap junctions by inhibiting the entry of calcium through voltage-dependent Ca^{2+} channels (Wang et al., 1990). Facilitatory effects of somatostatin in the production of LTP have been implicated in guinea pig hippocampus where this peptide significantly enhances potentiation in cysteamine- (a somatostatin depletor) treated animals (Matsuoka et al., 1991). In addition, a recent report of Wang et al. (1993) demonstrates that somatostatin specifically enhances glutamate responses mediated by NMDA receptors.

In conclusion, we have shown that somatostatin was colocalized with both glutamate and GABA neurotransmitters, suggesting a dual role for this peptide in the modulation of synaptic activity.

Figure 9. Localization of somatostatin in presynaptic endings synapsing on the lateral dendrite. *A*, A small vesicles bouton exhibiting preferential accumulation of somatostatin immunoreactivity (5 nm gold particles) over (solid arrowheads) DCVs. Note the presence of unstained DCVs (open arrowheads) and immunogold labeling over other axoplasmic compartments exemplified by arrows. *B*, General view of an LMCE displaying DCVs filled with (solid arrowheads) or devoid of (open arrowhead) somatostatin. Note the presence of sparse gold particles in the axoplasm and over (arrow) a mitochondrion (Mi). *C*, Higher magnification of region 1 of the LMCE shown in *B*. The labeled DCVs display gold particles associated with their rim (arrowheads). *D*, Another example from an SVB showing the preferential accumulation of gold particles on the rim of DCV (arrowhead). MC, Mauthner cell; *A_p*, Attachment plate; *, chemical excitatory synapse. Scale bars: *A*, 0.2 μ m; *B*, 0.5 μ m; *C* and *D*, 0.1 μ m.

Figure 10. Summary diagram showing the relative distribution of excitatory glutamatergic (GLU) LMCEs and inhibitory GABAergic (GABA) SVBs containing somatostatin (SOM) on distal parts of the lateral dendrite (LD). At this level (see box on the *M*-cell drawing), the relatively few SVBs containing only somatostatin also contain a second transmitter that, as with "empty profiles" elsewhere, may be glycine (see Colocalization of GABA and somatostatin). Toward the medial part of the dendrite, there are only GABAergic and glycinergic terminals intermingled with large vesicle boutons (LVBs) and small myelinated club endings (SMCEs). AC, S, VD, and VIII N, axon cap, soma, ventral dendrite, and eighth nerve, respectively.

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