Histol Histopath (1988) 3: 115-124

Histology and Histopathology

Intrinsic and extrinsic innervation of the amphibians esophagic myenteric plexuses

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Summary. The innervation of *Rana ridibunda* esophagus myenteric plexuses has been studied by the following methods: demonstration of cholinesterase activity; FIF method for catecholamines; immunohistochemistry for VIP, SP and SOM, and conventional electron microscopy.

The cholinergic innervation is important in the esophagus wall where, in addition to the well known extrinsic component, there is a rich intrinsic plexus with cells and fibres widely distributed.

The esophagus, together with the intestine, are the *Rana* gut portions where the adrenergic component is more broadly expressed. The adrenergic innervation seems to be almost entirely of extrinsic origin.

We have shown that, for the tested peptides, there is an intrinsic innervation represented by VIP, SP and SOM like plexuses. We do not discard nonetheless an extrinsic component.

The ultrastructure reveals the morphological characteristics of the enteric neurons as well as the fine inter-relationships between the nervous elements and the functional components of the esophagic wall.

Key words: Amphibians, Esophagic innervation, Myenteric plexuses

Introduction

Amphibians are interesting from the evolutionary point of view since is in the Anurans where, for the first time in vertebrates, appears a sacral parasympathetic system (Burnstock, 1969; Campbell, 1969). In the autonomic system of lower vertebrates the cholinergic system is dominant and in amphibians, for instance, it provides cholinergic and adrenergic innervation to smooth and cardiac muscles. In higher vertebrate forms, the adrenergic system will either replace the primitive parasympathetic system or will be added to it. It thus seems that, with the increasing vertebrates complexities, the adrenergic sympathetic innervation becomes more important and individualized.

With regard to the fibres content in amphibians, both systems, parasympathetic and sympathetic, present a mixed population of cholinergic and adrenergic fibres. The vagus nerve for instance has been considered as a vagus-sympathetic trunk. In the sympathetic pathways the adrenergic fibres proportion is very scarce in amphibians in comparison to the cholinergic one (Boyd et al., 1964; Norberg et al., 1967). The relative proportion of the adrenergic fibres in the sympathetic system increases as we approach to mammals. At the same time a progresive substitution at molecular level occurs: the adrenaline, typical of amphibians (Boyd et al., 1964), will be replaced by dopamine and noradrenaline which are typical of mammals (Pscheidt, 1963). Other fibres-classified as non-cholinergic and non-adrenergic have been postulated to be present. For Burnstock those fibres would be purinergic (Burnstock, 1969). Singh suggested the existence of «polinergic» fibres whose synaptic transmitters would change accordingly to the season, as a mechanism of poikiloterms seasonal adaptation (Singh, 1964).

On the other hand, the esophagus intrinsic innervation is represented by the intramural nervous plexuses -myenteric and submucosal in amphibians- which form the Enteric Nervous System (Gershon, 1981). Although it is generally believed that enteric neurons are not found in the submucous plexus of lower vertebrates Wong established, in 1971, the presence of cells in the submucous plexus of both levels, esophagus and stomach. Through their tinctorial characteristics it was considered that they could be either neurons or glial cells. We observed acetylcholinesterase positive cells in the submucous plexus of the frog esophagus and our ultrastructural

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results shown their neuronal nature (Junquera et al., 1987b).

As well as for the extrinsic component, non-cholinergic and non-adrenergic fibres were postulated to be present in the intrinsic innervation. We have considered that, at least in part, these fibres could be peptidergic.

Taking into account the above considerations, the aim of the present work was to study the *Rana ridibunda* cholinergic, adrenergic and peptidergic (VIP, SP and SOM) esophagus myenteric plexus components. We tried, in this way, to establish the distribution pattern of the cholinergic and adrenergic fibres in comparison with the peptidergic ones and to settle the neurochemical nature of the esophagus extrinsic and intrinsic innervation. At the same time, we shall compare our results with those from other vertebrates in order to establish the phylogenetic differences of the autonomic nervous system at the esophagus level.

Materials and methods

We have used 20 adult frogs in this study. The general histology of the esophagus wall was examined after staining with haematoxylin and eosin.

Acetylcholinesterase method (AChE)

Acetylcholine iodide (Sigma) was used for demonstrating the acetylcholinesterase (AchE) activity according to the El Badawi and Schenk (1967) method. The esophagus samples were frozen in methylbutane with liquid nitrogen and 30 μm sections were cut, air dried at room temperature and fixed for 15 min. at 4°C in a solution of 10% formaldehyde (commercial formaline) in PBS (pH 7.0). After washing in distilled water they were incubated for periods of 10-18hr at 37°C. The cholinesterase activity sites were recognized as dark brown precipitates. Controls were made by: (i) incubating in a substrate free medium, and (ii) incubating in a medium with tetraisopropylpyrophosphoramide (ISOOMPA, Sigma).

Catecholamines method (FIF)

The adrenergic fibres were visualized by the glyoxylic-formaldehyde method (Furness and Costa, 1975). The tissue was fixed by immersion in 2% glyoxylic acid in PBS (pH 7.4) for 3hr. It was then frozen in methylbutane with liquid nitrogen, freeze dried overnight or for periods up to 24hr, exposed to paraformaldehyde vapours (3hr at 80°C) and vacuum embedded in paraffin wax. Sections of 15-20 μm were examined under a Leitz-orthoplan fluorescence microscope. Adrenaline produces an apple green fluorescence, while histamine and serotonin produce a yellow fluorescence.

Immunohistochemical methods (IF)

We have used the method of indirect immunofluorescence applied on tissue sections and on whole mount preparations.

The method of Coons et al. (1955) has been applied on tissue sections. Esophagus pieces were fixed by immersion in 0.4% p-benzoquinone in PBS (pH 7.1-7.4.) for 3hr at 4° C. They were washed in a 7% sucrose solution in PBS and cryostat blocks were made in methylbutane immersed in liquid nitrogen. Sections of 20 μm were incubated in the primary antiserum for 24hr at 4° C in a humid atmosphere. After washing three times 5min. in PBS the positive reaction sites are recognized by incubating 1hr. at room temperature with fluorescein isothiocyanate-conjugated antibodies (FITC, dilution 1:20; Miles Lab. Ltd.). For control, non immune rabbit serum was used as first layer and the FITC globulin was used alone. We have tested the antisera: VIP (dilution 1:200 in PBS; pH 7.2-7.4; INC. Stillwater, MN); substance P (SP, 1:800) and somatostatine (SOM, 1:100).

We have introduced a modification to the method of Costa et al., 1980) to test the antisera on whole-mount tissue and to examine the three-dimensional distribution of the peptidergic fibres. Pieces of tissue were stretched and pinned on fine cork sheets. The fixation was made by immersion in a solution of 15% saturated picric acid with 2% formaldehyde in 0.1M PBS (pH 7.3) for 18hr at 4°C. After washing in 80% ethanol for 30min. The pieces were dehydrated through a graded series of ethanol and cleared in xylene, 30min. in each solution. The pieces were then rehydrated back to PBS. At this stage was made the delamination. We removed the mucous, submucous and inner muscular layers discovering the contact surface between the two muscle coats: the circular and the longitudinal one. We applied the antisera to both surfaces exposed since the plexuses not always remain attached to the longitudinal musculature. They were incubated for 16hr. at room temperature in a humid chamber. After three washes in PBS the pieces were incubated for 1hr. with the conjugated FITC. Then washed in PBS for 15min. and mounted in pure glycerol. The antisera dilutions used were those described above. The slides were kept overnight under pressure.

Electron microscopy method

The standard method for electron microscopy was applied: fixation in 2.5% glutaraldehyde in Buffer Milloning (pH 7.3), post-fixation in 2% OsO_4, stained with 70% uranyl acetate, dehydrated and embedded in araldyte. The ultrathin sections were contrast stained following the conventional methods.

Results

The haematoxylin-eosin method reveals that the histological organization of the *Rana ridibunda* esophagus shows some peculiar characteristics. The wall

has 6 to 8 longitudinal folds caused by the constriction of the esophagus. The mucosa epithelium is made by several layers of ciliated cells with intercalated goblet cells. Next to the cardioesophageal junction, the ciliated mucosae gradually change configurating a fundic structure typical of the stomach. The submucosa is made by lax connective tissue with esophageal glands, blood vessels and the nervous submucosal plexus. The muscularis mucosae, absent in the anterior portion, is organized near the stomach in bundles of cross-sectioned smooth muscle cells. The muscularis externa is very narrow in the anterior third of the esophagus increasing in thickness towards the stomach. At the proximal esophagus, the myenteric ganglia are located under the adventitia near the entrance of the blood vessels (Fig. 1a). As we approach to the stomach the ganglia will be gradually surrounded by the thin longitudinal musculature and then they reach their typical location, between the longitudinal and circular muscle coats (Fig. 1b). Thick nervous trunks are seen crossing the longitudinal musculature.

Cholinergic innervation

We have observed thick nervous axon bundles between the adventitia and the longitudinal musculature. The positive fibres for the AChE method are more abundant and more regularly distributed at the myenteric plexus where we have also got positive cells. The neurons appear either isolated or in small ganglia of 2 or 3 neurons. From these ganglia, medium size fibres leave branching abundantly in the circular musculature which is, in this way, more richly innervated than the longitudinal one (Fig. 2).

Adrenergic innervation

The esophagus longitudinal musculature adrenergic innervation is very scarce. Only in the circular muscle layer a rich varicose adrenergic fibre plexus is seen running in parallel to the muscle fibres. Very rarely positive adrenergic cells have been seen at the esophagic myenteric plexus (Fig. 3).

Peptidergic innervation

The immunohistochemistry method applied to the tissue sections allowed us to show varicose immunoreactive fibres in the myenteric plexus. Nonetheless the three-dimensional organization of the plexuses is much better defined in the whole mount preparations.

We have got positive reaction with VIP antiserum in cell bodies and fibres. The VIP immunoreactive cells are disposed either isolated at the points of the network intersections or in ganglia made by groups of 4 to 6 cell bodies, some ganglia lying near nerve bundle intersections, others being inserted in fibre bundles remote from intersections. The cells show an star shape with interconnected cytoplasmic branching (Fig. 4). VIP like fibres are seen in the connective tissue running in parallel to the smooth muscle fibres. The innervation density is higher at the circular layer than at the longitudinal one. At the level of the myenteric plexus varicose fibres (Fig. 5) of different diameters are arranged in wide networks with intersecting nerve bundles. This plexus is better developed in the posterior esophagus, near the stomach, than in the anterior one.

With the SP antisera we have observed immunoreactive cells, isolated or in small groups. They show a big nucleus (Fig. 6). Very tiny and isolated fibres are disposed in parallel to the muscle fibres in the proximal esophagus. Near the stomach the fibres density increases and the fibres are organized in fine networks (Fig. 7).

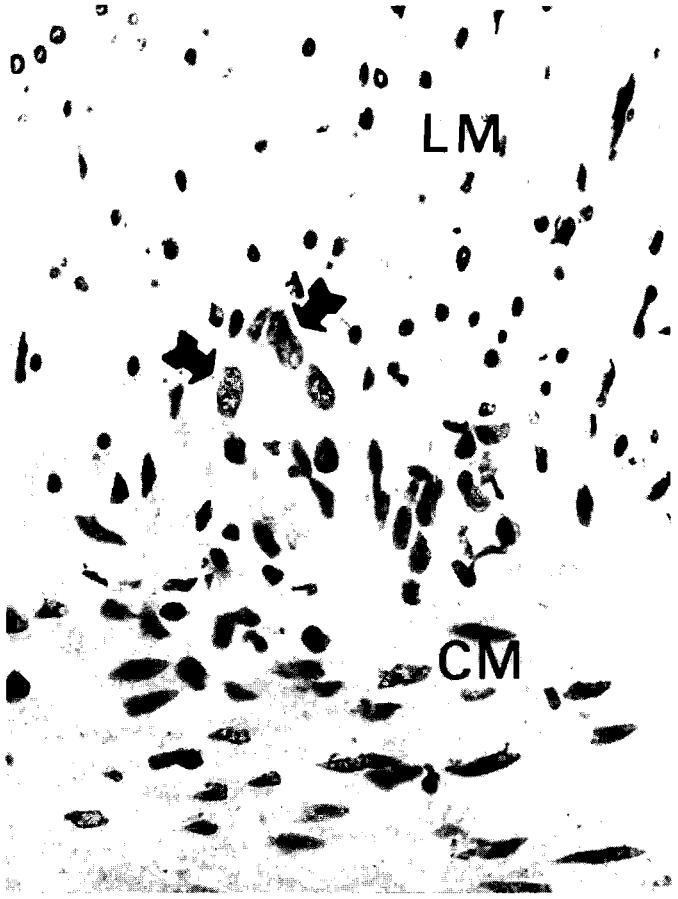
SOM positive cells are seen isolated in the proximal esophagus (Fig. 8). At the distal esophagus they appear in groups. Only rarely isolated SOM like fibres are seen isolated, they never make networks (Fig. 9).

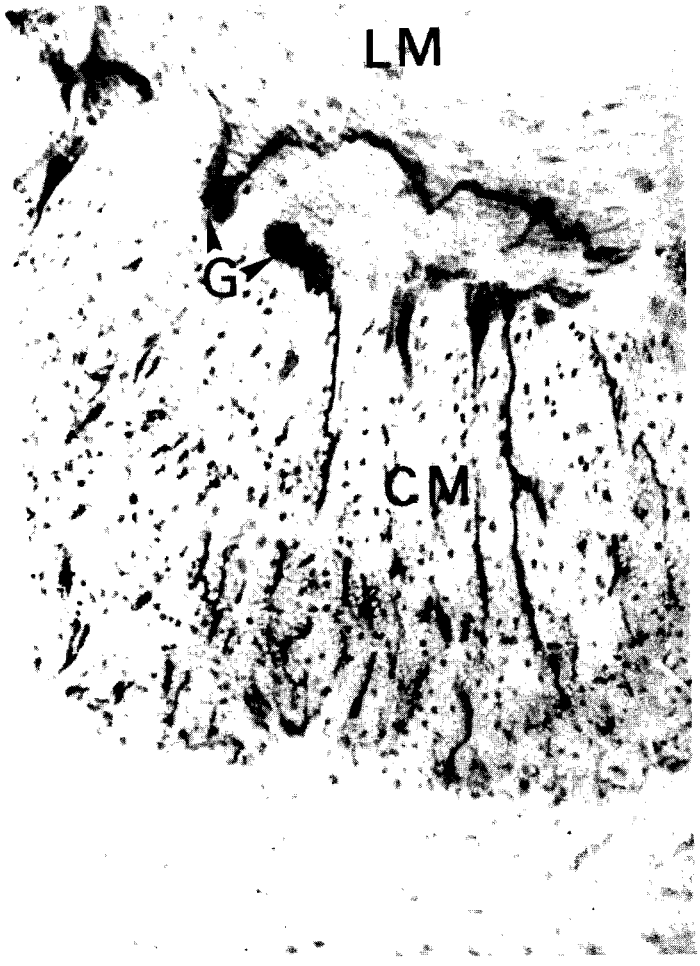
Ultrastructural results

The ganglia of the myenteric plexus show 2 or 3 neurons surrounded by the supporting glial cells (Fig. 10). The nuclei are oval shaped and rich in euchromatin with a fine marginal chromatin and patent nucleoli. The cytoplasm is rich in mitochondria and lipofuscine inclusions. In contact with the soma, varicosities charged with vesicles are seen, most of them of cholinergic type. Very often mixed varicosities are found with vesicles of cholinergic and peptidergic types. Groups of 4 to 6 axons with different varicosities types are disposed among the smooth muscle cells (Fig. 11). They are mostly of cholinergic or peptidergic type being the adrenergic ones very scarce. The mixed varicosities are prominent, with vesicles of cholinergic and peptidergic types.

Fig. 1a. Myenteric ganglia under the adventitia. (N: neuron; LM: longitudinal musculature.) H.E. × 25
Fig. 1b. Myenteric ganglia (arrows) between the longitudinal (LM) and circular (CM) muscle coats. H.E. × 40
Fig. 2. Cholinergic myenteric plexus. Tiny cholinergic positive fibres run toward the submucous layer (LM: longitudinal musculature; G: cholinergic ganglion cells; CM: circular musculature). AChE. × 10
Fig. 3. Adrenergic fibres in the circular muscle coat run in parallel to the muscle fibres. Positive cells (arrows) are very rarely seen at the myenteric plexus (LM: longitudinal musculature; CM: circular musculature). FIF. × 40
Fig. 4. VIP positive cells at the longitudinal musculature. IF. × 40
Fig. 5. Network of VIP like varicose fibres. IF. × 25
Fig. 6. SP positive cells. IF. × 80
Fig. 7. Network of SP like fibres. IF. × 25
Fig. 8. SOM positive cells. IF. × 25
Fig. 9. Network of SOM like fibres. IF × 25
Fig. 10. Ultrastructural organization of an intramural ganglion (N: neuron; CM: muscular cell; *: glial cells). × 3,800
Fig. 11. Axons group disposed near the muscle fibres showing varicosities with different vesicules types. × 15,000


N
LM


LM
CM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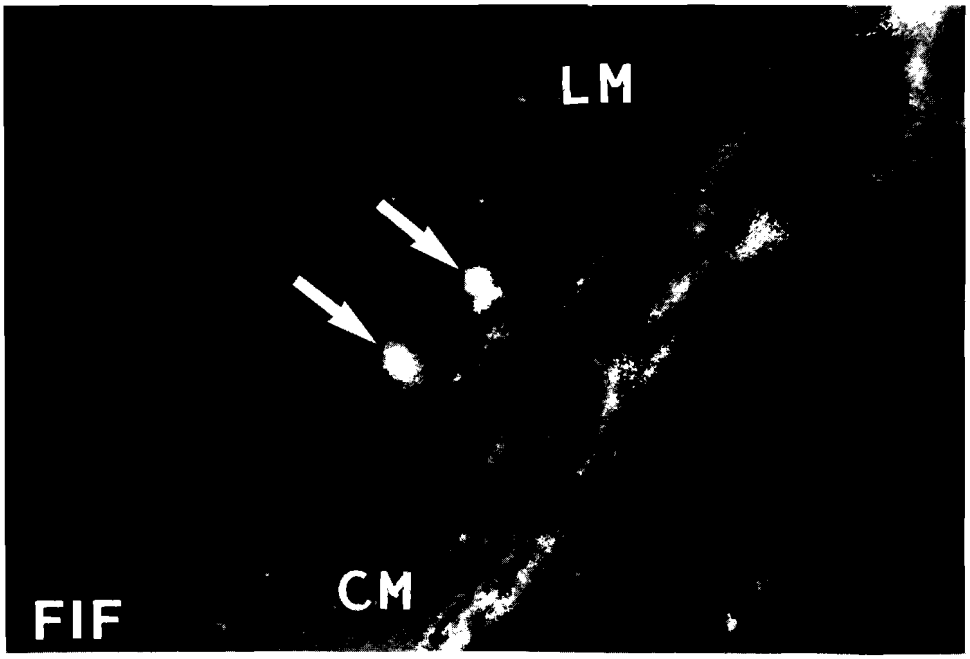
LM
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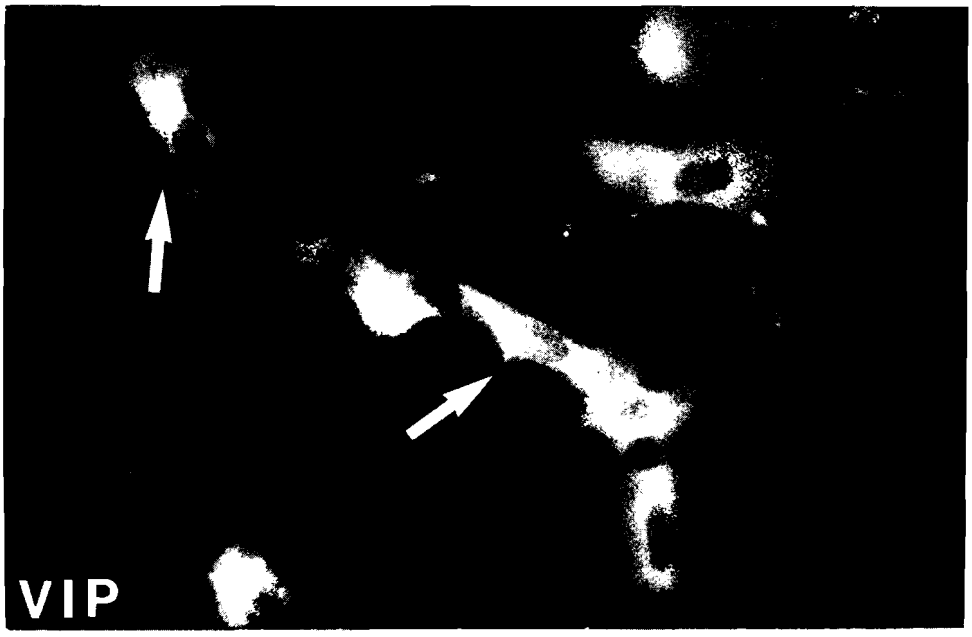
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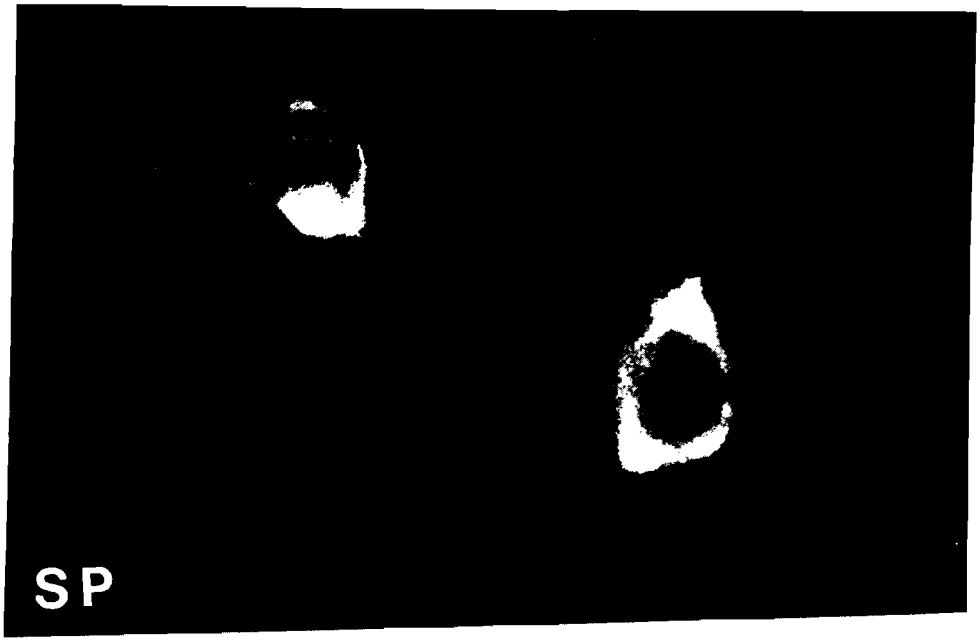
VIP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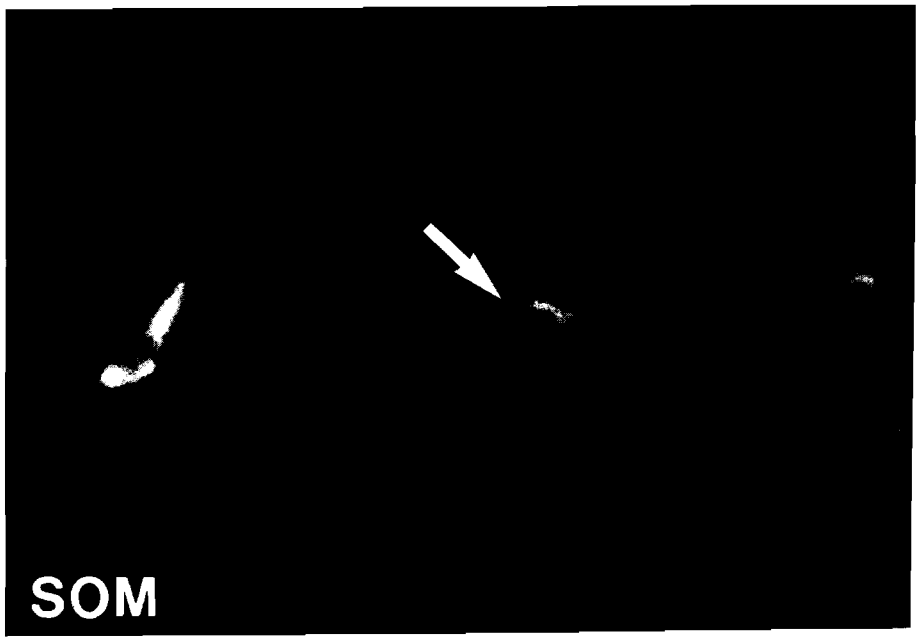
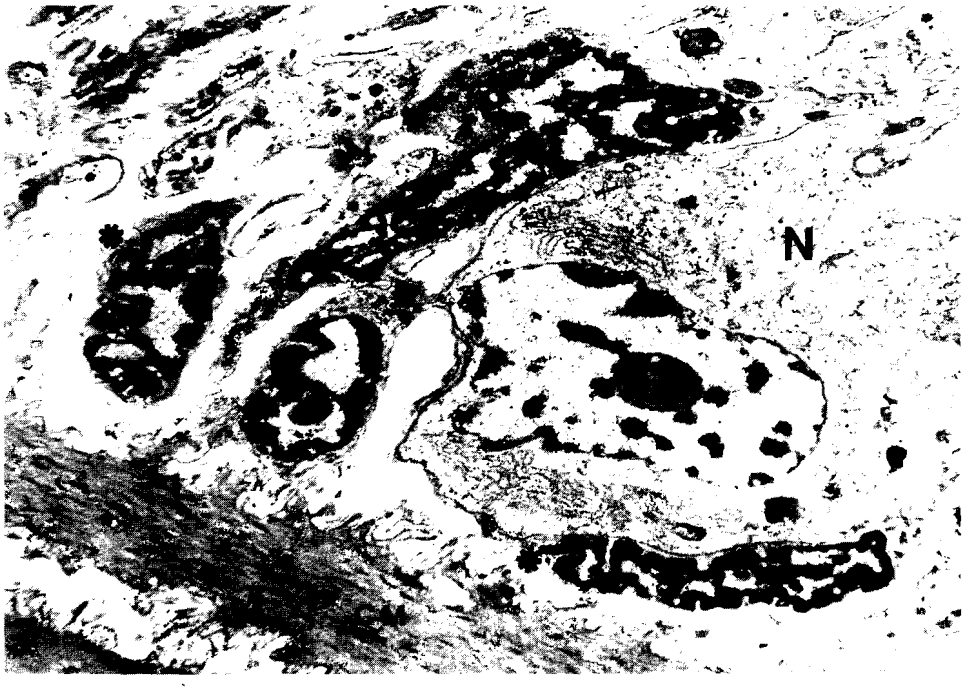
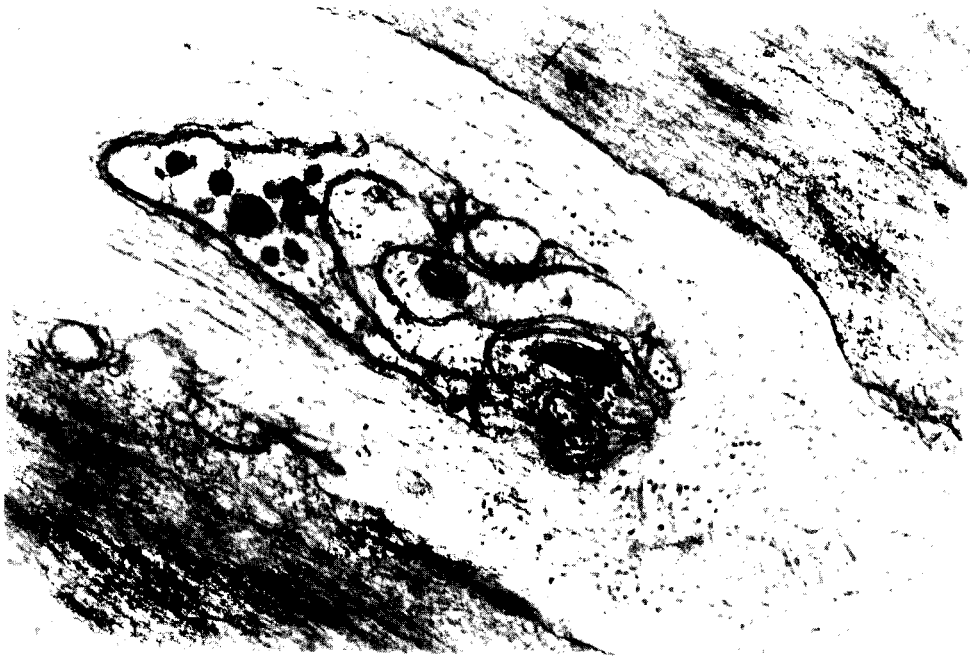
SP


SOM


SOM


N



Discussion

At the proximal esophagus the ganglia are located just below the adventitia as vagal paraganglia and it is at the medium esophagus where the ganglia get their typical location between the two external muscle coats. The esophagus myenteric ganglia are small. They are made by isolated neurons or by groups of 2 or 3 ganglion cells. In a description of the toad esophagic myenteric plexus, Wong (1973) reported bigger ganglia in *Bufo melanostictus* than the ones we have found in *Rana ridibunda.*

Although thick axon bundles are located between the adventitia and the longitudinal musculature, as revealed by the cholinesterase method, the former is poorly innervated. Exceptionally some fibres seem to distribute among the longitudinal muscle fibres. From the myenteric ganglia a rich fibre plexus branches abundantly in the circular musculature towards the submucosa. This plexus is mostly made by cholinergic and VIP like fibres. Our ultrastructural findings confirm our results at optic level, since it is shown that the profiles with acetylcholine vesicles type are the most frequent either isolated or in mixed varicosities with peptides. This is of particular interest since we know the inespecifity of the AChE method employed. Greenfield (1984) recently studying this problem decided that since the AChE appears also associated with non-cholinergic systems (adrenergic, dopaminergic, GABAergic, or SOM and SP), related with the Golgi apparatus or disolved in the axoplasma a new insight is necessary. He suggested that perhaps AChE is involved in the peptide processing or even as neuromodulator at the synapses. The problem is of course to see if the AChE not associated with cholinergic systems enclose unknown neural mechanisms.

In 1968 Read and Bunstock established that the main catecholaminergic neurotransmitter in amphibians is adrenaline whilst in reptiles and in mammals it is noradrenaline. This is also what we have got after 3hr. of exposition to the p-formaldehyde vapour. The adrenergic innervation distribution is not as uniform as the cholinergic one. It seems to be localized in certain tissue sections and requires the study of wide tissue portions.

The longitudinal musculature lacks adrenergic innervation, fact in common with the rest of Vertebrates including mammals (Read and Burnstock, 1969). The circular musculature is well supplied with adrenergic fibres, being this plexus as abundant as in the large intestine (Junquera et al., 1987a). The distribution of the adrenergic component in *Rana ridibunda* coincides with the data described for *Bufo marinus* (Read and Burnstock, 1969).

We have observed some adrenergic positive cells at the myenteric plexus of the esophagus as well as at the stomach and pylorus (Junquera et al., 1986), nonetheless we cannot decide about their nature or meaning. Read and Burnstock (1968) described, as an exception, adrenergic intramural neurons in the lizard large intestine. If we consider the phylogenetic position of amphibia, our results are not surprising. Cells of the same type have also been described in the guinea pig colon (Gabella, 1979). The function of these cells has not yet been established. It has even been suggested that they are not neurons (Kyosola, 1975).

Although the antisera tested had been developped against rabbit we have got reliable results in frogs.

The VIPergic like plexus is the best developed with many cell bodies in different locations. We have got more positive cell bodies in *Rana ridibunda* than Buchan et al. (1981) in *Rana temporaria*, in *Rana esculenta* and

other amphibia. On the other hand, in mammals the VIP-like cells seem to be restricted to the submucosal plexus (Jessen et al., 1980).

The substance P immunoreactivity has been poorer in comparison with that obtained with VIP. Our results contrast with those of Buchan et al. (1980) in *Salamandra* where they describe many SP like endocrine cells and fibres. We have observed isolated SP like neurons as in the intestine although they were more abundant in the last location (Junquera et al., 1987a). The SP like plexuses were poorly developed, only in the distal esophagus the fibres are arranged in fine networks. At the stomach wall (Junquera et al., 1986) we have not found SP like neurons, the fibres positive immunoreactivity was also scarce, only near the pyloric region their density increased.

The SOM plexus is not too developped in the esophagus myenteric plexus of *Rana ridibunda* . Buchan et al. (1981) have only described SOM like endocrine cells in the gut of *Salamandra*. In *Bufo regularis* it seems to be also restricted to endocrine cells (El-Salhy et al., 1981). We have nonetheless observed positive SOM like neurons isolated at the proximal esophagus or organized in small ganglia at the distal one. Although the SOM like plexus is not too developped at the esophagus the SOM component at this gut level is more important that in the stomach (Junquera et al., 1986). In comparison with the intestine, the peptidergic plexuses are much better developed in the large intestine than in the esophagus (Junquera et al., 1987a).

The connective tissue between the neurons and the supporting glial cells observed by electron microscope is similar to some reported mammal embrionic periods (Gabella, 1979). Although we have distinguished different neuron types in the frog esophagus it has been difficult to recognize exactly any of the 9 subdivisions made in the guinea-pig by Cook and Burnstock (1976). We neither can confirm the neurochemical nature of the cells since we have not made any specific study at the electron microscopy level. The circular smooth muscle coat is crossed by a high density of fibres. The profiles described here coincide with those found by Baumgarten et al. (1970) in several mammals. It is worth noting the high frequency of acetylcholine and peptides mixed profiles we have found prevailing. Our results at optic microscopy are confirmed ultrastructurally since we have shown that the different myenteric plexuses give branches towards the submucosal plexus.

We can consider that from a functional point of view all the neurotransmitters considered here, appart from having an important integration role in the main enteric plexuses must also have a direct influence on particular elements like vascular beds or smooth muscle cells regulating their specific activities.

Accordingly we can consider that the esophagic frog myenteric plexus is made by a mixed population of cholinergic, VIPergic and SP and SOM containing neurons. We cannot exclude the extrinsic origin of some of the observed nerve endings. The adrenergic component seems to be almost entirely of extrinsic origin.

Acknowledgements. The authors are indebted to Mr. C. Ulibarrena for technical assistance; to Mrs. M. Camuñas, E. Sanz and F. Camuñas for electron microscopical technical assistance and to Mrs. Marcellan for the careful photographic material development.

This research has been supported by the grant n° PB85/391 from the spanish CAICYT.

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Accepted September 10, 1987