ORIGINAL

A comparative study of the localization of vasoactive intestinal peptide- and substance P-like immunoreactivities in the submandibular salivary glands of mice

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Summary: The localization of vasoactive intestinal peptide (VIP)- and substance P (SP)-like immunoreactivities within the submandibular salivary glands (SMG) of Balb/c male mice at 4 to 5 weeks of age were investigated. Two immunocytochemical methods using polyclonal, monospecific antibodies, the indirect immunofluorescence and peroxidase-antiperoxidase (PAP), were used to elucidate the localization of VIP- and SP-like immunoreactivities in the SMG.

Both VIP- and SP-like immunoreactivities were positive in the SMG of Balb/c male mice at 4 to 5 weeks of age. The VIP-like immunoreactivity was found in the dense plexuses around the salivary duct and in the small scattered nerves surrounding glandular acini, and particularly in the nerve cell bodies of the submaxillary ganglion. The SP-like immunoreactivity was conspicuously abundant in the excretory ducts with a rich supply of SP-positive nerves, but also very scanty in the acinus areas close to the striated ducts and interlobular excretory ducts.

It could be conclusively observed that the localization of the VIP- and SP-like immunoreactivities were different in the submaxillary ganglion, in the nerves around blood vessels and acinus areas, but very similar in the areas of the excretory and striated duct of the SMG.

Introduction

Since von Euler and Gaddum¹⁾ have first discovered a factor which they designated as substance P (SP) present in equine brain and intestinal extracts, SP has been suggested to act as a sensory neurotransmitter with a wide range of biological effects, such as hypotensive, smooth muscle contracting and sialogenic actions²⁾. Subsequently, SP was reported to

be present in both the central and peripheral nervous systems as characterized immunocytochemically³⁾.

Since vasoactive intestinal polypeptide (VIP) was first isolated from porcine small intestine by Said and Mutt⁴⁾, VIP has also been shown to be present in both the central and peripheral nervous systems as a neuropeptide with a wide range of biological effects⁵⁾.

Although several reports are recently ac-

accumulated on the distribution of VIP- and SP-like immunoreactivities within the submandibular salivary glands (SMG) of various animals⁶⁻¹⁷, no information is available on the distribution of both VIP- and SP-like immunoreactivities within the SMG of mice except a report¹⁵. The present study was, therefore, designed to determine the localization of both VIP- and SP-like immunoreactivities within the SMG of mice, using polyclonal, monospecific antibodies.

Materials and Methods

Specimens

Specimens of the SMG were collected from ten Balb/c male mice at 4 to 5 weeks of age. Mice were obtained from Institute for Experimental Medicine, Military Academy, Belgrade, Yugoslavia. The mice were perfused with 0.4 % solution of benzoquinone in 0.01 M phosphate buffered saline (PBS, pH 7.1-7.4) for 5 min. Immediately, they were killed by cervical dislocation and the SMG was fixed in the 0.4 % benzoquinone solution for 2 h. Subsequently, the tissue was rinsed several times in a solution containing sucrose (7 %) and sodium azide (0.1 %) in PBS. After cryostat blocks were prepared, sections were cut at 10 to 15 μ m, collected on poly-L-lysine (PLL)-coated slides and processed for the immunostaining.

Antisera

Antisera used were kind gifts from the Histochemical Department, RPMS, Hammer-smith Hospital, London, UK. Their sources were as follows:

1. Anti-C-terminal directed antisera to the natural and synthetic porcine VIP were raised in rabbits and described in detail by Wharton *et al.*⁶.
2. Polyclonal, monospecific antisera to SP were described and used by Wharton *et al.*⁶ and Polak *et al.*¹⁸.

These antisera did not cross-react with other known peptide hormones in the immunocytochemical procedures (personal communication with Prof. J.M. Polak).

Second- and third-layer antibodies were as follows: Goat anti-rabbit FITC conjugated antibodies (Zymed Lab., Inc., San Francisco, CA), goat anti-rabbit IgG (H+L) (Miles

Lab., Inc., Elkhart, IN) and rabbit peroxidase-antiperoxidase (PAP, Miles Lab., Inc., Elkhart, IN).

Immunostaining

The indirect immunofluorescence¹⁹ and PAP²⁰ immunostaining methods were used. The color was visualized by 3, 3'-diaminobenzidine tetrahydrochloride (DAB)²¹. After developing, the sections were counterstained with methylgreen, mounted in 80 % glycol/PBS and examined by an Olympus AH-2-microscope. Comparative photographs were taken using Kodak Ectachrome 50 film (50 ASA).

Controls

Negative controls were as follows:

- a. The tissue sections were directly incubated with each antiserum without any following antibody steps.
- b. The first- or second-layer was omitted, or the first-layer was replaced with normal goat sera.
- c. Antisera pre-absorbed with their respective peptides (0.1-10 nmol/ml of diluted antiserum) were applied.

Results

Nerve fibers containing VIP- and SP-like immunoreactivities were observed in the SMG of Balb/c male mice at 4 to 5 weeks of

Fig. 1 The VIP-like immunoreactivity in the SMG of mice.

In the SMG of Balb/c male mice, the VIP-like immunoreactivity was detected in ganglion cell bodies and nerve fibers around the excretory duct and big blood vessels by the PAP method. Arrow denotes VIP-like immunoreactive nerve cell bodies of the submaxillary ganglion. Magnification = $\times 100$

Fig. 2 The VIP-like immunoreactivity in the SMG of mice.

In the SMG of Balb/c male mice, dense plexuses of nerves rich in the VIP-like immunoreactivities (Fig. 2A), small scattered nerves around the acini (Fig. 2B) and the strong immunostaining of the nerve cell bodies of the submaxillary ganglion (Fig. 2C) were observed by the indirect immunofluorescence method. Arrow in Fig. 2C denotes VIP-like immunoreactive ganglion nerve cell bodies. Magnification = $\times 100$.

age. Two staining protocols, such as the PAP and indirect immunofluorescence methods, provided good results. In addition, successful quenching of the immunostaining was obtained by prior absorption of each anti-

Fig. 3 The SP-like immunoreactivity in the SMG of mice.

In the SMG of Balb/c male mice, the excretory duct received a rich supply of the SP-like immunoreactivity, was observed by the PAP method. Magnification = $\times 100$.

serum with its respective antigen, such as 0.1 nmol/ml of synthetic VIP (Sigma, V3628) or 2 nmol/ml of synthetic SP (Sigma, E8254).

VIP-like immunoreactivities were detected both in the dense plexuses of nerves around the excretory duct (Fig. 1, and Figs. 2A and B), and in the small scattered nerves surrounding the glandular acini and running to the small blood vessels (Fig. 2B). The most peculiar findings were strong VIP-like immunoreactivities in the nerve cell bodies of the submaxillary ganglion (Fig. 2C). This was obvious also after the the PAP immunostaining (Fig. 1, arrow). A bunch of thin VIP-like immunoreactive nerves originated from this ganglion nerve cell body was observed (Fig. 2C, arrow). All controls were negative.

The excretory ducts received a particularly rich supply of SP-like immunoreactive nerves, highly obvious with the PAP immunostaining (Fig. 3), but also with the indirect immunofluorescence staining (Figs. 4A and B). The distribution of SP-like immunoreactivities within the SMG was markedly patchy in the areas surrounded by nerves (Figs. 4A and B). This was particularly conspicuous within the acinus areas close to the striated ducts (Fig. 4B) and interlobular excretory ducts (Fig. 4A). However, acinus areas do not lack SP-like immunoreactive nerves (Fig. 4C). Contrary to the VIP-like immunoreactivities, the submaxillary ganglion nerve cell bodies, tiny nerves within the gland and

Fig. 4 The SP-like immunoreactivity in the SMG of mice.

In the SMG of Balb/c male mice, the patchy distribution of the SP-like immunoreactivity (Figs. 4A and B), and strong SP-like immunoreactive nerves close to the interlobular excretory (Fig. 4A) and striated ducts (Fig. 4B), but the distant area totally devoid of the immunoreactivity (Fig. 4 B, arrow) can be seen. Acinus areas are innervated with SP-like nerves (Fig. 4C). The indirect immunofluorescence method was used. Arrow in Fig. 4B denotes lack of SP-like immunoreactivities in the acinus areas. Magnification = $\times 100$.

blood vessels do not show any SP-like immunoreactivity. All controls were not positive.

Discussion

The existence of nerves histologically distinct from the classical cholinergic and adrenergic types was first postulated by Taxi²²⁾ and later established in detail by Baumgarten *et al.*²³⁾. This new kind of nerve was referred to peptidergic (p-type). In the salivary gland, p-type neurosecretory granules have recently been elucidated by Kidd *et al.*²⁴⁾. The discovery of highly active regulatory peptides in the autonomic and sensory nerves of a wide range of peripheral organs, has dramatically changed old ideas of a bipartite autonomic nervous system⁹⁾. The salivary glands are not exceptional, as they contain significantly high contents of such biologically active peptides. The VIP-, originally isolated from small intestine by Said *et al.*⁴⁾, and SP-like immunoreactivities were detected in the nerve fibers of the SMG of Balb/c male mice at 4 to 5 weeks of age, regardless of the two staining protocols.

For the presence of the VIP-like immunoreactivities, our data were consistent with the several reports, as described by several investigators in the SMG of rats^{6,8,16)}, cats^{6,8,11)}, humans^{6,8)} and mice¹⁵⁾, and in the sublingual glands of rats^{6,9)} in general. However, in the SMG of Balb/c male mice at 4 to 5 weeks of age, an abundant supply of VIP-positive immunostainings was seen in the nerves around the salivary duct, between the glandular acini and along the small blood vessels, and in the nerve cell bodies of the submaxillary ganglion, but not within the cells of duct or acinus portions. There are small differences of the intensity of VIP-like immunoreactivities in the salivary glands among different animal species^{6,8)}. These results on the distribution of VIP-like immunoreactivities in the SMG were also very similar to those in the human and rat pancreas²⁵⁾.

The origins of VIP-containing nerves in the SMG are not so far clear, but it could be postulated that they derived principally from an intrinsic neuronal system^{6,9)}, which is still unknown definitely, and in a lesser degree from the postganglionic parasympa-

thetic nerves^{6,9,16,26}. An intrinsic neuronal system of VIP-containing nerve fibers should be checked by further studies in detail. A similar intrinsic system has also been postulated in SP-containing nerve fibers of the SMG of rats by Goedert *et al.*¹². However, VIP-like immunoreactivities, as well as SP-like immunoreactivities, are also found in neurons of sensory ganglia or in the dorsal horn of the spinal cord²⁷.

For the presence of the SP-like immunoreactivities in the salivary glands, there are some reports on the distribution in the SMG of rats^{7,10,12,17}, in the parotid glands of rats¹⁴, dogs and mice¹⁰, and in the sublingual glands of rats^{13,17}. Although all organs, in which effects of SP had been shown, also contained the SP-like immunoreactivities, as demonstrated by the immunocytochemical or radio-immunoassayable methods¹⁰, the parotid and the pooled SMG and major sublingual glands contained the highest levels of the SP-like substances among all tested organs of rats¹⁰. No report on the distribution of the SP-like immunostaining is available in the SMG of mice. However, present results could confirm the presence of the SP-like immunostaining in the SMG of mice (Figs. 3 and 4). In the SMG of mice, the patchy and varicose SP-like immunoreactivities were observed around the excretory and striated ducts and between the acini, although the SP-like immunoreactivities were not found around the blood vessels and within the nerve cell bodies of the submaxillary ganglion. Our results were consistent with those obtained in the SMG of rats^{12,13}.

The dual origins of the SP-positive nerve fibers are postulated in the SMG of rats¹². One type of the SP-like immunoreactivities in the SMG of rats originates from the intrinsic system and locates around the acini, as reported on the VIP-like immunoreactivities^{6,9}, whereas another type of them originates from the sensory neurons and locates mainly around the major ducts of the SMG, which is affected by neonatal capsaicin treatment, as confirmed in the heart²⁸, the iris²⁹, the tongue³⁰ and the dental pulp³¹. It is very worthwhile to know that non-capsaicin sensitive nerves containing the SP-like im-

munoactivities are predominantly present in the SMG of rats¹², in contrast that VIP-like immunoreactivities are mainly present in post-ganglionic parasympathetic nerve fibers and coexist with acetylcholine^{15,26}.

On the other hand, both endocrine-like and exocrine cells contain the SP-like immunoreactivities resistant to capsaicin treatment, as confirmed in the gastrointestinal tract³² and SMG¹². Since the physiological roles and their origins of the intrinsic SP as well as the intrinsic VIP are yet unclear, further studies are, therefore, necessary to elucidate them definitely. The correlation between SP- and VIP-like immunoreactivities has already been examined in the dental pulp by double immunofluorescence³³ and the different distribution profiles of VIP- and SP-positive nerve fibers were observed in the same blood vessels within the same sections of the dental pulp³³. In order to elucidate the co-existence of VIP- and SP-like immunoreactivities in the SMG of mice, further studies are necessary to be carried out by using the dual immunostaining methods, since very similar results were observed between VIP- and SP-like immunoreactivities in the excretory and striated ducts, but not in the submaxillary ganglion, blood vessels and acinus portions of the SMG of mice.

To elucidate the physiological roles of these neurotransmitters or neuromodulators was not our aim in this paper, but it may appear that these peptides exert various actions which could be synergistic or antagonistic, as mentioned by Polak *et al.*³⁴, even in the salivary glands of mice. The complexities and a wide variety of functions of the neuroendocrine system in exocrine organs should further be elucidated in their integrities in both autonomic and sensory nervous systems in the salivary glands of animals.

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抄録 : 4 ~ 5 週齢の Balb/c 系雄マウスの顎下腺を用いて, vasoactive intestinal polypeptide (VIP) と substance P (SP) の局在を各種のポリクロナール抗体を用いた PAP 法および間接蛍光抗体法から比較検索した。VIP および SP 陽性物質はともにマウスの顎下腺に存在していた。VIP 陽性物質は導管系周囲の神経叢, 腺房細胞を取り囲むように存在する散在性の小神経線維, 血管壁に沿って走る小神経線維および顎下神経節の神経細胞体に認められた。神経節では特に顕著であった。

一方, SP 陽性物質は SP 陽性神経が豊富に存在していると思われる外導管部に顕著に認められたが, 線条部や小葉間導管部に近接する腺房細胞には少ししか認められなかった。VIP と SP 陽性物質の局在は線条部や外導管部では非常に類似していたが, 顎下神経節や腺房部では異なっていた。また, 血管周囲の染色性にも両者間に相違が認められた。

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