Immunohistochemical localisation of regulatory neuropeptides in human circumvallate papillae

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ABSTRACT

The occurrence and distribution of neuropeptide-containing nerve fibres in the human circumvallate papillae were examined by the peroxidase–antiperoxidase immunolocalisation method using surgical specimens that had not been subjected to radiotherapy, and the abundance of neuropeptide-containing fibres was expressed as the percentage of total nerve fibres demonstrated by protein gene product (PGP) 9.5 immunoreactivity for a quantitative representation of these peptidergic fibres. Substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactive (IR) nerve fibres were densely distributed in the connective tissue core of the circumvallate papillae, and some SP and CGRP-IR fibres were associated with the taste buds. A moderate number of vasoactive intestinal polypeptide (VIP)-IR fibres and a few galanin (GAL)-IR fibres were also seen in the connective tissue core and subepithelial layer. There were, however, no VIP-IR or GAL-IR fibres associated with the taste buds. Neuropeptide Y (NPY)-IR fibres were few and were associated with the blood vessels. Within the epithelium of the circumvallate papillae, no peptidergic fibres were found, although a number of PGP 9.5-IR fibres were detected. The abundance of SP, CGRP, VIP, and GAL-IR fibres expressed as the percentage of total PGP 9.5 IR fibres was $25.35 \pm 3.45\%$, $22.18 \pm 3.26\%$, $10.23 \pm 1.18\%$, and $4.12 \pm 1.05\%$, respectively. The percentage of NPY-IR fibres was below 3%. In a deeper layer of the papillae, a few VIP, GAL, and NPY-IR ganglion cells were found, and VIP immunoreactivity was detected in a few cells of the taste buds. There was no somatostatin, leucine enkephalin, or methionine enkephalin immunoreactivity in the circumvallate papillae. These results suggest that the dense SP and CGRP-IR fibres within the connective tissue core of the human circumvallate papillae may be involved in the deep sensation of the tongue.

Key words: Tongue; taste buds; neuropeptides; protein gene product (PGP) 9.5.

INTRODUCTION

In mammals, substance P (SP) immunoreactive (IR) nerve fibres form complicated networks within the connective tissue core of circumvallate and fungiform papillae, and some of them are associated with the taste buds (Lundberg et al. 1979; Nagy et al. 1982; Nishimoto et al. 1982; Yamasaki et al. 1985; Astbäck et al. 1997). In addition, calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), and galanin (GAL) (Baecker et al. 1983; Terenghi et al. 1986; Luts et al. 1990; Montavon et al. 1991; Montavon & Lindstrand, 1991*a*, *b*; Nosrat et al. 1996; Astbäck et al. 1997) IR nerve fibres have been found within these papillae. In birds, VIP-IR fibres have also been reported in the quail tongue (Baecker et al. 1983). In amphibians, immunoreactivity of SP, CGRP, VIP, NPY and GAL is localised in nerve fibres distributed in the filiform papillae of the bullfrog tongue in addition to the fungiform papillae (Hirata & Kanaseki, 1987; Kuramoto, 1988; Kusakabe et al. 1996). Thus the nerve fibres innervating the lingual papillae of various vertebrates, from amphibians to mammals, contain

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several regulatory neuropeptides, although the occurrence of peptidergic fibres in the filiform papillae was restricted to the amphibian tongue. As far as we are aware, there is only a single immunohistochemical report concerning to the peptidergic innervation of the human lingual papillae (Astbäck et al. 1995), and no immunohistochemical studies on the peptidergic innervation in the human circumvallate papillae are available.

In the present study, 8 neuropeptides, SP, CGRP, VIP, NPY, GAL, somatostatin (SOM) and leucine and methionine enkephalins (l-ENK and m-ENK) were examined immunohistochemically in the circumvallate papillae of human tongues which had not undergone radiotherapy, because Forsgren et al. (1992) and Franzén et al. (1991) have recently suggested that irradiation causes fluctuation of neuropeptide immunoreactivity. In addition, the immunoreactivity of neuropeptides was compared with protein gene product (PGP) 9.5 immunostaining, which is an effective immunohistochemical marker for the peripheral nervous system in man (Wilson et al. 1988), for a quantitative expression of these peptidergic fibres.

MATERIALS AND METHODS

Tissue preparation

Seven circumvallate papillae were obtained from a total of 5 male patients (aged 37, 48, 49, 52 and 60 y) with tongue cancer. None of the patients had been treated with radiotherapy before operation. The surgical tissue samples were immediately fixed by immersion in 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer saline (PBS), pH 7.3, at 4 °C. Under a dissecting microscope, normal circumvallate papillae, which were macroscopically free from malignant infiltration, with surrounding tissues were taken from these specimens, sliced longitudinally into 2 blocks, and immersed in the same fixative for 8 h. Dissection was carried out in the cold fixative within a few minutes. After a brief washing with PBS, the specimens were transferred to 30% sucrose in PBS and kept there overnight at 4 °C. The specimens were then sectioned at 20 μm on a cryostat, and mounted on poly-L-lysine coated slides. To confirm whether the conditions of fixation were satisfactory, and whether the tissue samples had been invaded by malignant cells, selective sections taken from all samples were stained with haematoxylin and eosin, and only samples which were not invaded with malignant or inflammatory cells were processed for immunohistochemistry. We have previously confirmed that this protocol is sufficient to obtain well-fixed human tissues for reliable immunohistochemistry (Kusakabe et al. 1997; Matsuda et al. 1997).

Immunohistochemistry

The sections were processed for immunohistochemistry according to the peroxidase-antiperoxidase (PAP) method. Before PAP treatment, sections were dipped in a fresh 0.3% solution of hydrogen peroxide in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. After washing in several changes of 0.3% Triton-X in 0.1 M PBS (PBST), the sections were treated for 1 h with a protein blocking agent (Immunon, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4 °C overnight with rabbit polyclonal antisera against the following peptides: SP (Cambridge Research Biochemistry, UK, 1:2000), CGRP (Cambridge, 1:1500), VIP (Incstar, USA, 1:1500), NPY (Incstar, 1:1500), GAL (Cambridge, 1:1500), SOM (Incstar, 1:1500), l- and m-ENKs (Incstar, 1:1000). Some sections were also incubated with antiserum for PGP 9.5 (UltraClone, UK) to identify the total population of nerve fibres. The antisera were diluted with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST. After rinsing in several changes of PBST, the sections were transferred for 2 h to antirabbit IgG (Organon Teknika, USA) diluted to 1:200 with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST at room temperature. Next the sections were rinsed with several changes of PBS, transferred for 2 h to rabbit PAP complex (Jackson, USA) diluted to 1:200 with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% thimerosal in PBS, and rinsed in several changes of PBS. The peroxidase activity was demonstrated with 3,3′-diaminobenzidine. This immunostaining procedure has been detailed in a previous report (Kusakabe et al. 1991).

Controls

Primary antisera were first incubated with an excess (50–100 μM) of each respective peptide. The absorbed antiserum was then used for incubation of the section followed by incubation with the secondary antiserum to test the specificity of the primary antisera.

Data analysis

To compare the abundance of peptidergic fibres, their length was measured with an ARGUS 100 computer and image processor (Hamamatsu-Photonics, Japan) on 50 randomly sampled fields taken from 7 circumvallate papillae of 5 patients, and the length of immunoreactive fibres within the epithelium and in the connective tissue core of the papillae was calculated. The abundance of intra- and subepithelial nerve fibres immunoreactive for neuropeptides was expressed as the percentage (mean $\pm$ S.D.) of total nerve fibres demonstrated by PGP 9.5 immunoreactivity.

RESULTS

Figure 1*a* shows the total population of neural elements as labelled by PGP 9.5 immunoreactivity in a vertical section of a circumvallate papilla. A dense network of relatively thick nerve bundles covered the whole connective tissue core of the papilla. Relatively thin bundles were also seen in the secondary papillae. A number of fine fibres with many varicosities penetrated into the epithelium of the papilla (Fig. 1*b*). In the lateral epithelium, all neural elements and cells within the taste buds were also stained with PGP 9.5 outlining the barrel-shaped taste buds (Fig. 1*b*). Many intragemmal PGP 9.5-IR nerve fibres ascended up to the taste pore (Fig. 1*b*). Some small clusters of PGP 9.5-IR cell bodies were also detected in a deeper layer of the papilla (Fig. 1*c*).

SP and CGRP IR nerve bundles, which are composed of several thin immunoreactive fibres with many varicosities, were numerous in the connective tissue core of the circumvallate papillae (Figs 2*a*, 3*a*). When a pair of adjacent sections, one immunostained with SP antiserum and the other with CGRP antiserum, was observed, the distribution of most SP-IR fibres was similar to that of CGRP-IR fibres (Figs 2*a*, 3*a*). These varicose fibres were also seen in the secondary papillae. In the subepithelial layer, a few thin fibres penetrated into the taste buds. Except for these intragemmal fibres, there were no immunoreactive fibres within the epithelium (Figs 2*b*, 3*b*), although some SP and CGRP-IR fibres were detected in the stratified epithelium around the papillae.

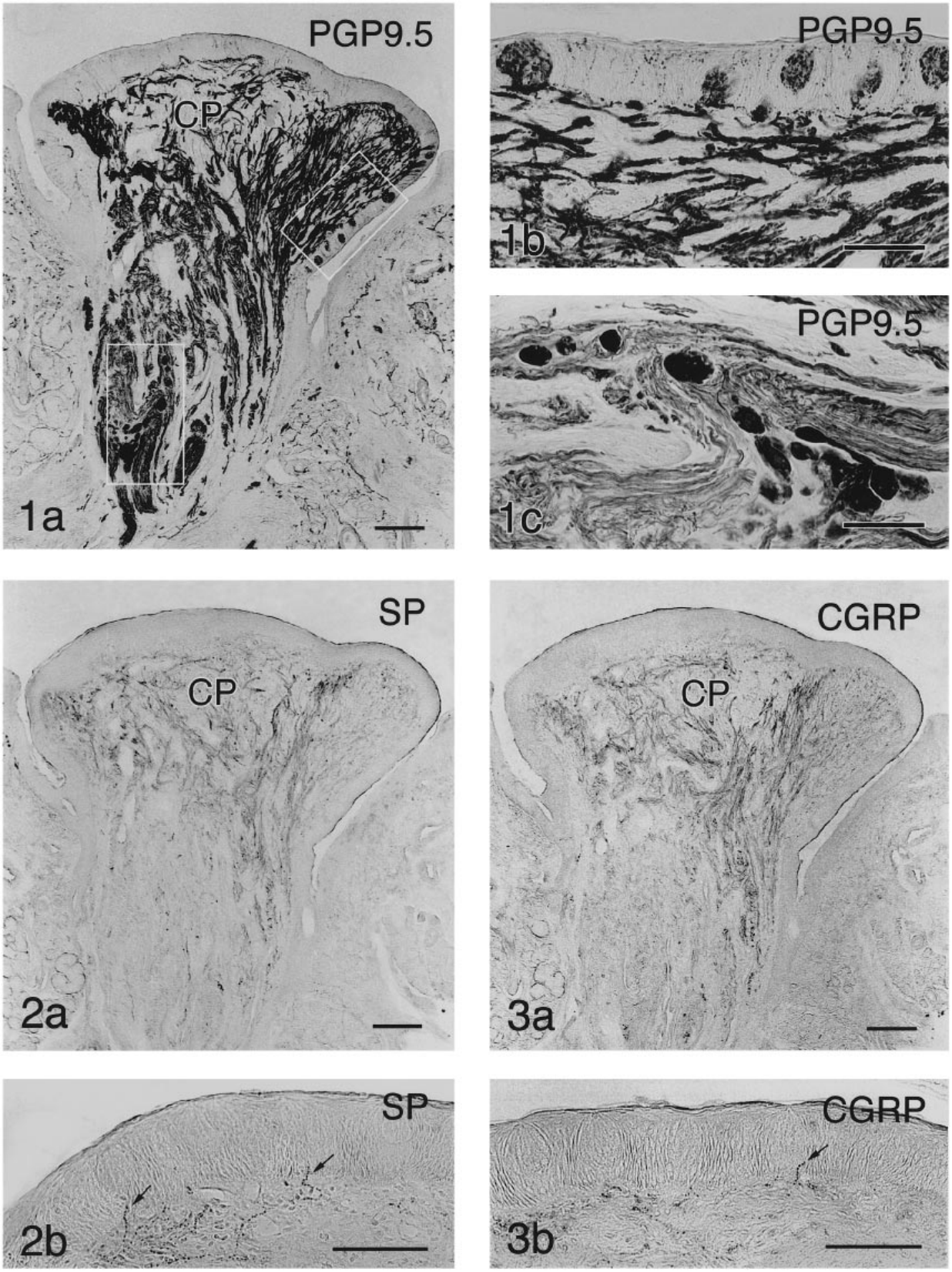
A moderate number of VIP-IR varicose fibres were distributed in the connective tissue core of the papillae without forming thick nerve bundles (Fig. 4). Some VIP-IR fibres were associated with the small blood vessels. A few GAL-IR fibres were also seen in the subepithelial layer (Fig. 5). No VIP-IR or GAL-IR fibres were detected within the epithelium. NPY-IR fibres were few, and were mainly found along blood vessels distributed in the core (Fig. 6). There were no SOM, l-ENK, or m-ENK-IR fibres in the papillae.

When the frequency of neuropeptide immunoreactive fibres in the connective tissue core of the papillae is expressed as the percentage of total PGP 9.5-IR fibres, the abundance of SP, CGRP, and VIP fibres was $25.35 \pm 3.45\%$ ($n = 50$), $22.18 \pm 3.26\%$ ($n = 50$) and $10.23 \pm 1.18\%$ ($n = 50$), respectively. That of GAL-IR fibres was $4.12 \pm 1.05\%$ ($n = 50$), and that of NPY-IR fibres was below 3% ($n = 50$). When the frequency of peptidergic fibres within the epithelium is expressed in the same way, the frequency of SP and CGRP associated with the taste buds was below 2% ($n = 50$). There was no distinct difference in the frequency of peptidergic fibres between 7 circumvallate papillae examined. The frequency of the 8 neuropeptides is summarised in the Table.

In a deeper layer of the papillae, some clusters of VIP, GAL, and NPY-IR ganglion cells were found (Figs 7, 8, 9). No SP (Fig. 2*a*), CGRP (Fig. 3*a*), SOM, l-ENK, or m-ENK-IR cells were detected. On the other hand, VIP immunoreactivity was found in a few cells of the taste buds (Fig. 10). VIP cells were usually located singly between nonimmunoreactive cells. There were no taste bud cells immunoreactive for the other 7 neuropeptides. The occurrence of the immunoreactive ganglion cells and taste bud cells is also summarised in the Table.

DISCUSSION

In the human circumvallate papillae, the present study has demonstrated, for the first time, the occurrence and distribution of several kinds of neuropeptide-containing nerve fibres and VIP-containing taste bud cells, although Astbäck et al. (1995) have recently reported the peptidergic innervation in the human fungiform papillae. Comparing the results with those from the human fungiform papillae, the occurrence and distribution of SP, CGRP, VIP, NPY and GAL-containing nerve fibres in the connective tissue core and in the epithelium of both papillae resembled each other. There were, however, some differences in the abundance and distribution of these peptidergic fibres. In general, the density of these peptidergic fibres in the human circumvallate papillae was higher than that in the human fungiform papillae. In addition, although the distribution pattern of SP fibres was different from that of CGRP fibres in the fungiform papillae (Astbäck et al. 1995), in the present findings of a pair of adjacent sections of the human circumvallate



Figs 1*a*, 2*a*, 3*a*. Three serial vertical sections of a human circumvallate papilla (CP).

Fig. 1. (*a*) Protein gene product (PGP) 9.5-IR nerve fibres in the circumvallate papilla (CP). In the lateral epithelium, many PGP 9.5 fibres and cells are found in the taste buds, and in a deeper layer of the papilla, PGP 9.5 IR small ganglion cells are seen. (*b*, *c*) Enlargement of area in *a*. Bars: *a*, 200 μm; *b*, *c*, 100 μm.

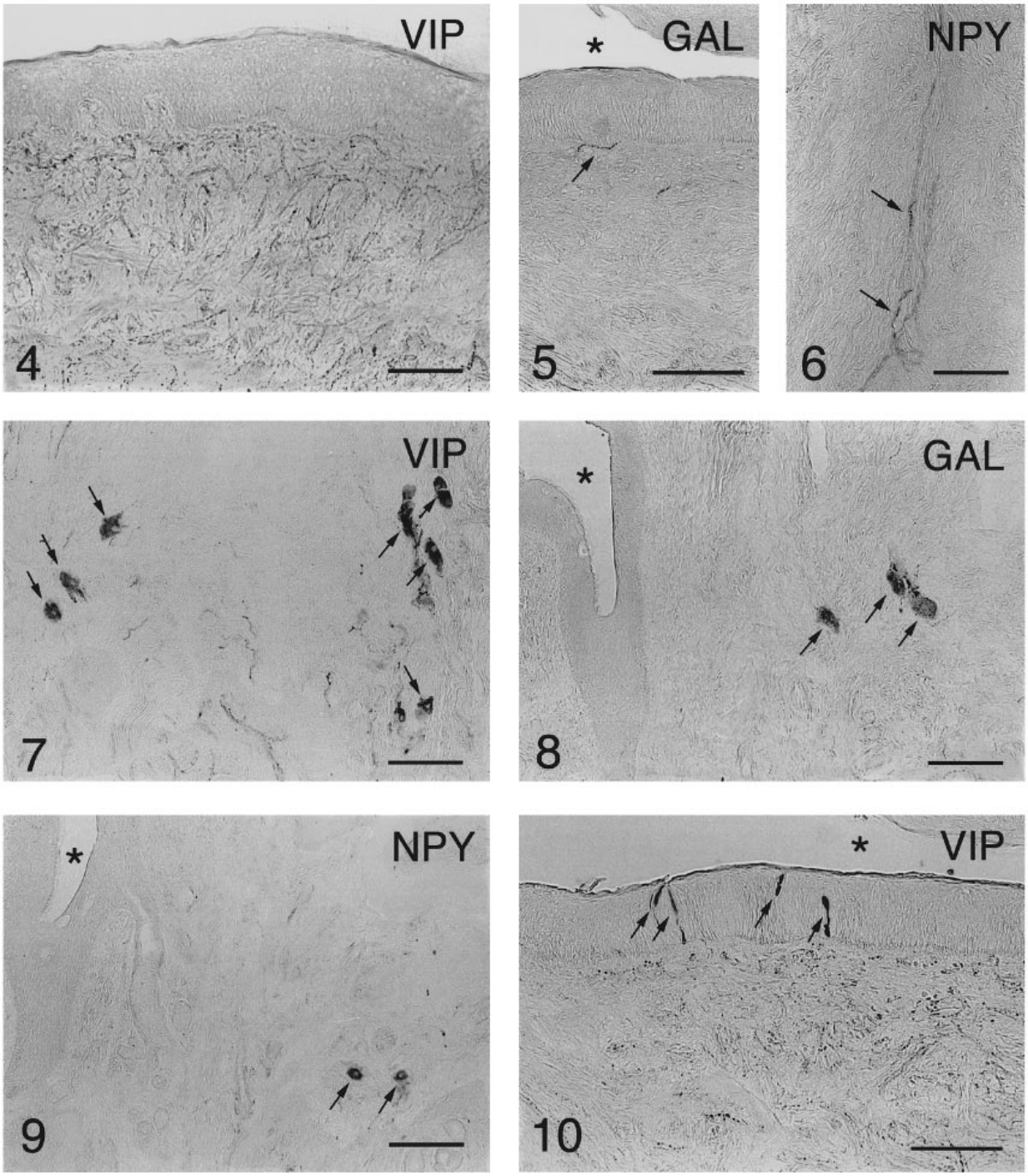


Fig. 4. Vasoactive intestinal polypeptide (VIP)-IR fibres in a circumvallate papilla. Bar, 100 μm.

Fig. 5. Galanin (GAL)-IR fibres (arrow) in the subepithelial area. Asterisk indicates a deep circular furrow. Bar, 100 μm.

Fig. 6. Neuropeptide Y (NPY)-IR fibres (arrows) along a blood vessel running through the connective tissue core of the papilla. Bar, 100 μm.

Figs 7–9. VIP (Fig. 7), GAL (Fig. 8), and NPY (Fig. 9) -IR local ganglion cells in a deeper layer of the papillae. Asterisks indicate the circular furrow. Bars, 100 μm.

Fig. 10. VIP-IR taste bud cells (arrows). Bar, 100 μm.

Fig. 2. (*a*) Substance P (SP)-IR nerve fibres in a circumvallate papilla (CP). (*b*) Higher magnification image of the lateral epithelium of CP. Two arrows indicate SP fibres associated with the taste buds. Bars, 100 μm.

Fig. 3. (*a*) Calcitonin gene-related peptide (CGRP)-IR nerve fibres in a circumvallate papilla (CP). (*b*) Higher magnification image of the lateral epithelium of a CP. Arrow indicates CGRP fibres associated with a taste bud. Bars, 100 μm.

Table. *Frequency of peptidergic nerve fibres and occurrence of neuropeptide immunoreactive local ganglion cells and taste bud cells in human circumvallate papillae*

	SP	CGRP	VIP	GAL	NPY	SOM	leu-ENK	met-ENK
Intraepithelium	–	–	–	–	–	–	–	–
Intragemmal	1.95 ± 0.43	1.77 ± 3.45	–	–	–	–	–	–
Connective tissue core	25.35 ± 3.45	22.18 ± 3.26	10.23 ± 1.18	4.12 ± 1.05	2.36 ± 0.62	–	–	–
Local ganglion cells	(–)	(–)	(+)	(+)	(+)	(–)	(–)	(–)
Taste bud cells	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(–)

Results are expressed as the percentage (mean ± S.D.) of total nerve fibres demonstrated by PGP 9.5 immunoreactivity (n = 50).
–, absent. Occurrence of immunoreactive cells is represented by (+), present, or (–), absent.

papillae, the distribution of SP fibres was similar to that of CGRP fibres. This difference in the abundance and distribution of the peptidergic fibres may be related to that for the innervation of these 2 papillae. Taste buds of the fungiform papillae are innervated by the chorda tympani and those of the circumvallate papillae by the glossopharyngeal nerve; the somatosensory innervation of anterior parts of the tongue is through the lingual nerve, a branch of the trigeminal nerve, and the posterior parts are innervated by the glossopharyngeal nerve (Mistretta, 1991).

Furthermore, comparing the findings of human circumvallate papillae with those of experimental animals such as the monkey, pig, cow, ferret, cat, rat, mouse and frog (for references see Introduction), it appears that SP, CGRP, and VIP-IR fibres were predominant in the connective tissue core of the circumvallate papillae of all animal species including man. There was, however, a difference in the occurrence of SP and CGRP-IR fibres within the epithelium of the papillae. In the experimental animals, many intraepithelial SP and CGRP-IR fibres were detected, but there were no neuropeptide-containing fibres within the epithelium of the human circumvallate papillae except for a small number of intragemmal SP and CGRP-IR fibres, in spite of the dense distribution of PGP 9.5-IR fibres within the epithelium. This indicates that no intraepithelial fibres of the human circumvallate papillae contain SP and CGRP, and that only a small number of intragemmal fibres contain these 2 peptides. The surgical specimens used in this study were taken from patients who had not received radiotherapy, and were immediately transferred into fixative. Consequently, any influence of irradiation and poor fixation can be discounted. As evidence for this, some SP and CGRP-IR fibres were detected within the stratified epithelium around the papillae. In the human circumvallate papillae, consequently, all SP and CGRP-IR fibres in the epithelium are associated with the taste buds. It is likely that the intraepithelial nerve fibres in the human circumvallate papillae are nonpeptidergic sensory fibres originating from the glossopharyngeal nerve. However, there are 2 other possibilities that need to be considered. The intraepithelial fibres may contain other neuropeptides besides those examined in this study, and the other peptides investigated, except for SP and CGRP may be below the limit of detection because of the small amount of the peptide in relation to the sensitivity of the technique. Although these problems remain unsolved, the sensory mechanisms in the epithelium of the human circumvallate papillae may differ from those of experimental animals because human food habits are different. In addition, small VIP, GAL, and NPY-IR ganglia were seen in the human lingual parenchyma. This suggests that at least some of the VIP, GAL, and NPY-IR fibres in the connective tissue core of the papillae may originate in these local ganglia.

The physiological role of neuropeptide-containing nerve fibres in the lingual papillae has not been elucidated, in spite of their dense distribution. In rats, it has been speculated that SP fibres associated with the taste buds may participate as a trophic factor, because the SP-IR synapses do not make contact with the taste cells (Yamasaki et al. 1984). In contrast, Finger (1986) suggested that SP modulates the taste bud activity. In the present study, the ratio of intragemmal SP and CGRP-IR fibres to the intragemmal PGP 9.5 fibres was extremely low ($< 2\%$). Accordingly, the small number of SP and CGRP fibres associated with the taste buds in the human circumvallate papillae may not be involved in these 2 possible roles. On the other hand, the widespread distribution of SP and CGRP in the connective tissue core of the circumvallate papillae may suggest an involvement in deep sensation in these papillae. It is necessary to obtain further pharmacological data to clarify this.

In the mammalian vascular system, many physiological studies have suggested the vasoactive nature of VIP and NPY in association with vascular smooth

muscles. VIP (Heistad et al. 1980; Wilson et al. 1981) is thought to have a vasodilator effect, and NPY (Lundberg et al. 1982; Edvinsson et al. 1983) is thought to have a vasoconstrictor effect. On this basis, we believe that these immunoreactive fibres found around the periphery of the blood vessels are probably involved in controlling local circulation.

Nearly 100 y ago, nerve fibres terminating near the taste pore were reported (Arnstein, 1893; Botezat, 1902; Retzius, 1912). Recently Müller (1996) and Kanazawa & Yosie (1996) reexamined this by methylene blue supravital staining and by immunoreactivity for PGP 9.5, respectively. Müller (1996) proposed 2 possible roles for the nerve fibres near the taste pore: (1) a paracellular pathway in taste transduction; and (2) regulation of the taste pore aperture diameter directly via the nerve terminals. The latter phenomenon has also been observed after exposure to chemical stimuli (Mattern & Paran, 1974). In the taste buds in the human circumvallate papillae, many PGP 9.5 fibres were detected near the taste pore, although they were not immunoreactive for the 8 neuropeptides examined. Even granted that these 2 speculations hold true for human taste buds, it seems that these mechanisms have no relation to neuropeptides.

VIP IR cells have been detected in the taste buds of the foliate and circumvallate papillae in rat and hamster tongues (Herness, 1989), and in the oral cavity of teleost fishes such as carp and catfish (Witt, 1995). According to these reports, most cells in the rodent taste buds are immunoreactive for VIP, whereas in the bony fishes, only taste cells are immunoreactive for VIP. The distribution pattern of VIP cells in human taste buds was similar to that in teleost fishes, although it is not clear whether the VIP cells in the human taste buds are taste cells or not. The physiological role of intracellular VIP in taste reception remains unclear.

In conclusion, SP, CGRP, VIP, NPY, and GAL IR nerve fibres were distributed in the human circumvallate papillae. Considering together with the recent study of the human fungiform papillae reported by Astbäck et al. (1995), the abundance of these fibres in the circumvallate papillae were more frequent than that in the fungiform papillae. The widespread distribution of SP and CGRP-IR fibres within the connective tissue core of the circumvallate papillae may be involved in the deep sensation in the tongue.

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