Substance P-Immunoreactive Neurons of the Bovine Forestomach Mucosa: Their Presumptive Role in a Sensory Mechanism

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Summary. Substance P-immunoreactive nerve fibers and cell bodies were examined in the forestomach mucosa of the calf and cow pretreated with colchicine, using thick (100 μm) floating sections. Intraepithelial nerve fibers were identified, appearing only rarely in the rumen and reticulum, and completely absent from the omasum. Nerve fibers were observed in the lamina propria of all the regions of the forestomach examined. A few thin nerve fibers were seen in the core of the ruminal papillae of the calf, whereas in the cow they appeared very coarse in nature. Flocculent and complicated nerve fiber networks were seen in the connective tissue of the reticular papillae. Mucosal nerve fibers formed a peculiar glomerulus-like architecture in the omasal papillae of the calf, while in the cow, the nerve fibers were largely restricted in distribution to the vicinity of the epithelium within the connective tissue pegs. Immunoreactive nerve cell bodies were found in the ruminal atrium, the dorsal sac and the ventral sac of the rumen of the calf and in the reticulum of both the calf and cow. Some of these neurons exhibited processes that appeared to course toward the papillae. In total, substance P-immunoreactive nerve fibers and cell bodies were more abundant in the calf than in the cow.

The ruminant forestomach consists of three compartments: rumen Rumen, reticulum Reticulum and omasum Omasum (Fig. 1). Each compartment has peculiar mucosal protrusions: ruminal papillae Papilla ruminis, reticular fold Crista reticuli exhibiting a honeycomb-like appearance (reticular cell Cellula reticuli) studded with reticular papillae Papilla reticuli, and omasal lamina Lamina omasi studded with omasal papillae Papilla omasi. All of these protrusions are covered with cornified stratified squamous epithelium, and yet absorption and secretion occur. The reticular groove Sulcus reticuli functions to prevent milk from entering the rumen and reticulum of the suckling calf.

The regulatory mechanism of this complicated stomach has been investigated extensively, with the majority of studies focusing on the vagal or extrinsic regulation of the muscular function. In the course of these studies, it has been suggested that mucosal nervous elements may play a role in sensory reception (mechano- and chemo-receptors) of the forestomach (LEEK and HARDING, 1975; LEEK, 1986). Intrinsic nerve reflexes via the enteric nervous system of the ruminant forestomach in response to chemical stimuli from the lumen also have been proposed (GREGORY, 1982, 1984, 1987; COTTRELL and GREGORY, 1991).

Nerve fibers supplying the mucosa and/or muscle layers of the ruminant forestomach have been demonstrated by the silver impregnation method (HABEL, 1956; GRAU and WALTER, 1957; ABE, 1959; ABE et al., 1959). However, neither intraepithelial nerve fibers nor sensory receptors other than free endings could be demonstrated by these techniques or electron

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microscopy (STEVENS and MARSHALL, 1970; SCHENK-SAUBER et al., 1985). Although histological and histochemical methods such as methylene blue perfusion, cholinesterase staining and the osmium-zinc-iodate method demonstrated intraepithelial nerve fibers (HILL, 1959; COMLINE and MESSAGE, 1965; SCHENK-SAUBER et al., 1985), no ganglia were observed in the lamina propria mucosa or tela submucosa. An extensive review and experiment by HABEL (1956) has summarized the controversy as to the presence of intraepithelial nerve fibers and mucosal/submucosal ganglia in the ruminant forestomach.

Immunohistochemistry has been used to study peptide-containing nerves in the stomach of cattle (KITAMURA et al., 1986, 1987a, b) and sheep (WATHUTA, 1986; WATHUTA and HARRISON, 1987; WEYNS et al., 1987). Although previous studies had described the distribution of mucosal nerve fibers, these studies about peptide-containing nerves concentrated primarily on the muscle layer and the myenteric plexus.

The present study utilizes substance P-immunoreactivity as an immunohistochemical marker for nervous elements, as substance P-immunoreactive nerve fibers are one of the most abundant types of peptide-containing nerves reported in the forestomach mucosa of cattle (KITAMURA et al., 1986, 1987a). Sensory nerves in the digestive tract are generally considered to contain substance P (SHARKEY et al., 1984; FURNESS et al., 1988; BORNSTEIN et al., 1989; COSTA et al., 1991). The present study demonstrates substance P-immunoreactive nerve cell bodies and nerve fiber networks in the forestomach mucosa of cattle.

A part of the present study has previously appeared in a preliminary form (KITAMURA et al., 1989).

**MATERIALS AND METHODS**

Two Holstein calves (10- and 15-days-old) and a cow (8-years-old) pretreated with colchicine (5 mg/kg, intraperitoneally) were used in the present study. Tissue samples were obtained from the rumen (dorsal sac Saccus dorsalis, ventral sac Saccus ventralis, atrium Atrium ruminis, and pillar Pila ruminis), reticulum, reticular groove and omasum (Fig. 1). The tissues were fixed with either a mixture of picric acid and formalin or Bouin's fluid for 20-30 h. After washing with 70% alcohol and 0.1 M phosphate buffered saline (PBS, pH 7.4), the tissues were embedded in 30% gelatin and cut at 100 μm in thickness using a Microslicer (Dosaka, Kyoto). These sections were degelatinized with PBS and washed with 0.5% Triton X-100 in PBS for 48 h. After washing with PBS, the sections were treated with 0.3% H2O2 for 30 min followed by further washing with PBS, and then treated with normal rabbit serum (1:50) for 1 h. Floating sections were then stained immunohistochemically using an anti-substance P monoclonal antibody (Sera-Lab, Sussex, clone NC1/34HL, Lot. B6E35, 1:1,000) for 48 h, a biotinylated anti-rat IgG antibody (Vector, Burlingame, 1:400) for 2 h and a Vectastain Elite ABC kit (Vector, 1:4) for 2 h. Immunoreactive sites were visualized by diaminobenzidine and H2O2 in Tris-HCl buffer. Immunostained sections were then mounted on poly-L-lysine coated slides and air dried. They were dehydrated with ethanol, cleared with xylene and cover-slipped.

Specificity of the immunostaining was verified either by replacement of the first antibody with antibody pretreated with an excess amount of substance P (Peptide Institute, Osaka) or omitting one of the staining steps. Occasional non-specific coloring of the cornified stratified squamous epithelium could not be prevented.

**RESULTS**

Substance P-immunoreactive nerves were demonstrated in all regions of the bovine forestomach examined. Both immunoreactive nerve fibers and cell bodies of individual sampling sites were far more abundant in the calf than in the cow. The smooth muscle area in the lips of the reticular groove, the muscle layers, the reticular folds and the omasal laminae contained a relatively abundant number of substance P-immunoreactive nerve fibers in comparison to other layers of the forestomach wall. The myenteric ganglia contained numerous substance P-immunoreactive nerve cell bodies as well as moderate numbers of immunoreactive nerve fibers. The immunoreactive nerve cell bodies frequently occupied a peripheral location in the myenteric ganglia. Numerous perivascular nerve fibers in the forestomach wall showed substance P-immunoreactivity. These results confirm and extend our previous observations (KITAMURA et al., 1986, 1987a). However, the features of the distribution of immunoreactive nerves were remarkably different in the mucosa. Therefore, the present paper focuses on the immunoreactive nerves in the mucosa, as these have yet to be considered.

**Rumen**

Substance P-immunoreactive nerves were more numerous in the atrium than in other parts of the rumen of both the calf and cow. Positive nerve fibers were far more abundant and densely distributed in
Fig. 1. Schematic drawings showing regions (1–7) of the forestomach of the calf and the cow from where the specimens were sampled. 1 Dorsal sac of the rumen, 2 ventral sac of the rumen, 3 ruminal pillar, 4 atrium of the rumen, 5 reticular groove, 6 reticulum, 7 omasum. RuP ruminal papilla, Li lip of the reticular groove, Fl floor of the reticular groove, ReF reticular fold, ReP reticular papilla, LOL large omasal lamina, SOL small omasal lamina, OP omasal papilla. The ruminal mucosa does not contain a lamina muscularis mucosae. The reticular mucosa does contain smooth muscle bundles in the upper part of the reticular fold, but these are not connected with the muscle layers. The omasum contains a continuous sheet of the lamina muscularis mucosae. The lips, Labium dextrum et sinistrum, of the reticular groove contain muscular protrusions continuing from the inner muscle layer of the reticulum. Large and small arrowheads point to the esophagus and the pylorus, respectively. The asterisk indicates the abomasum, glandular stomach, which was not examined in the present study.
Fig. 2. Substance P-immunoreactive nerve fibers and a nerve cell body in the ruminal mucosa.  

**a.** Nerve fibers in the ruminal papilla taken from the ruminal atrium of the calf. ×240.  
**b.** A nerve fiber in the core of a ruminal papilla taken from the ruminal atrium of a cow. E identifies the covering of cornified stratified squamous epithelium. ×240.  
**c.** An intraepithelial nerve fiber (arrow) demonstrated in the dorsal sac of the adult rumen. The broken line indicates the basal border of the stratified squamous epithelium. ×480.  
**d.** A smooth surfaced nerve cell body (arrow) and nerve fibers in the subepithelial layer of the dorsal sac of the calf. ×240
the calf than in the cow. In the calf, the nerve fibers in some ruminal papillae often appeared organized into a particular network (Fig. 2a), whereas in the cow, fine varicose fibers were observed occasionally in the core of the ruminal papillae (Fig. 2b). Many of the fibers in the ruminal papillae of the cow were associated with vascular elements although a few were seen beneath the epithelium. Some parts of the rumen which contained none or very short ruminal papillae, such as the ruminal pillar and the dorsal sac, showed only a very few positive fibers primarily in the connective tissue pegs beneath the epithelium. On very rare occasions nerve fibers were demonstrated in the stratified squamous epithelium of the calf atrium and in the adult dorsal sac (Fig. 2c).

In the calf, positive nerve cell bodies were found in the lamina propria close to the epithelium, though this was a rare observation (Fig. 2d). Nerve cell bodies were more frequent in the atrium, rare in the dorsal sac, and absent from the ventral sac or the pillar region. No positive nerve cell bodies were found in the ruminal mucosa of the cow. The nerve cell bodies of the calf usually were seen as scattered solitary cells, although on rare occasions a small aggregate of a few cells was observed. Some exhibited branching processes that appeared to extend toward the ruminal papillae. Many of the nerve cell bodies in the ruminal mucosa were round or oval in shape with a smooth external surface, giving them a unipolar or pseudounipolar appearance (Fig. 2d).

**Reticular groove**

Numerous nerve fibers were found in the muscle layer of the lips of the reticular groove. This region contained the greatest abundance of nerve fibers in the forestomach of both the calf and cow (Fig. 3a). Nerve fibers were more numerous in the calf than in the cow. Subepithelial nerve fibers were more extensively developed in the floor of the groove than in the lips, being less numerous in the floor than in the other parts of the forestomach. Nerve fibers radiated in a broom-like fashion into the papillae located on the floor of the reticular groove of the calf (Fig. 3b). Ganglia containing immunoreactive nerve cell bodies were usually seen in the lips of the reticular groove (Fig. 3a). Immunoreactive nerve cell bodies appeared only rarely in the mucosa and submucosa of the lips and in the muscle layer of the floor in the calf; they were not observed at all in the cow.

**Reticulum**

Substance P-immunoreactive nerve fibers were seen predominantly in the reticular papillae, with many being associated with the overlying epithelium. Several nerve fibers were observed passing up the lamina propria core of the reticular papillae on the floors of the reticular cells and on the reticular folds. These fibers branched many times, became convoluted, and took on a flocculent or broom-like appearance in the core of the papillae (Figs. 3c, d, 4). These complicated structures became more extreme at the tip of the reticular papilla. Side branchings of fibers were observed on occasion, these extending to other areas including the interpapillar space and/or the lamina propria core of other papillae. The proportion of the papillae containing highly developed neural networks seemed to be greatest in the floor of the reticular cell in comparison to those located on the reticular fold. With regard to the reticular fold, the papillae containing condensed fiber networks appeared more numerous at the top of the fold than on the side walls of the fold. Nerve fibers were identified in the muscle bundles of the reticular fold (Fig. 5a) as well as at the base of the fold which contained no smooth muscle bundles (Fig. 5b). Although intraepithelial nerve fibers were observed at the top of the reticular fold, they were too few in number to be examined with regard to their pattern of distribution (Fig. 5c).

Substance P-immunoreactive nerve cell bodies were located predominantly in the floor of the reticular cell (Fig. 4a, b) and appeared to be more numerous in the calf than in the cow. The nerve cell bodies located in the floor of the reticular cell seemed to innervate two or more adjacent reticular papillae (Fig. 4a). Moreover, nerve cell bodies located at the base of the reticular fold seemed to be related to the flocculent neural networks in the papillae on the summit of the reticular fold, via fibers passing through the smooth muscle bundles in the reticular fold (Fig. 4b). In the calf, mucosal nerve cell bodies were present singly or aggregated into small clusters of several cells (Fig. 5d). In the cow, aggregations of two or more nerve cell bodies were seen only on rare occasion. The mucosal nerve cell bodies had a smooth external surface with few processes, giving these cells a unipolar or pseudounipolar appearance. Occasional nerve cell bodies were found in the smooth muscle bundles within the reticular fold of the calf, often being localized in the crossing region of the fold (Fig. 5a). Nerve cell bodies were found only in the base of the reticular fold of the cow (Fig. 5b).

**Omasum**

Numerous nerve fibers were seen in the mucosa
Fig. 3  

a. Substance P-immunoreactive nerve fibers and nerve cell bodies in the lip of the reticular groove from a calf. G indicates a ganglion showing the peripheral arrangement of nerve cell bodies. M indicates the muscle layer in the lip. ×67. 

b. Substance P-immunoreactive nerve fibers showing the broom-like branching in a papilla on the floor of the reticular groove from a calf. ×110. 

c and d. Nerve fibers in the reticular papillae on the floor of a reticular cell from a cow. c: ×67, d: ×110
Fig. 4. Substance P-immunoreactive nerve cell bodies and fibers in the reticulum. a. Nerve fibers and cell bodies (arrow) in the floor of a reticular cell from a calf. x 110. b. Nerve fibers in the reticular fold and nerve cell bodies (arrows) at the base of the fold from a calf. x 95. c. Nerve fibers in the reticular papilla at the summit of a reticular fold from a cow. x 75
Fig. 5. Substance P-immunoreactive nerve fibers and nerve cell bodies in the reticulum.

a. Nerve fibers in smooth muscle bundles and nerve cell bodies (arrows) at the crossing of the reticular fold of the calf viewed in a horizontal section. ×75.

b. Nerve fibers and nerve cell bodies (arrows) in the base of a reticular fold of a cow. The reticular fold does not contain smooth muscle bundles at this height. Horizontal section. ×75.

c. An intraepithelial nerve fiber (arrow) at the top of a reticular fold of a calf. ×570.

d. Substance P-immunoreactive ganglia seen in the mucosa, being the floor of a reticular cell from a calf. ×240
Fig. 6. Substance P-immunoreactive nerve fibers in the omasum. **a.** Nerve fibers are especially abundant in the omasal laminae of the calf. *Arrowheads* indicate some of omasal papillae containing abundant nerve fibers. ×17. **b.** Abundant nerve fibers in the core of the omasal papillae present a glomerulus-like appearance. They seem to originate from nerve fibers in the muscle layer (m) of the omasal lamina. ×100. **c.** Nerve fibers form a delicate network in the omasal papilla of the cow when viewed in a tangential section. ×67. **d.** Subepithelial nerve fibers in the connective tissue pegs observed along the side of a omasal papilla of a cow. ×95
covering the omasal laminae. There were marked differences in distribution patterns between the calf and cow. In the calf, one or more thick nerve bundles, running from the muscle bundles in the omasal laminae, formed a glomerulus-like or a fountain-like network in the core of the omasal papillae (Fig. 6a, b). In contrast, a lattice-like network of nerve fibers was seen in the omasal papillae of the cow (Fig. 6c). Moreover, nerve fibers ran in the vicinity of the epithelium, especially within the connective tissue pegs (Fig. 6d). Subepithelial nerve fibers seen in the cow were often branched and followed a tortuous and recurrent course, wandering among the connective tissue pegs close to the epithelium. They were few in number in the core of the omasal papillae. Mucosal nerve fibers were more abundant in the calf than in the cow, and were more numerous on the oral side than on the aboral side and in the free border than in the base of the laminae. Mucosal nerve networks of the calf and the cow tended to be more complicated on the oral side, although not all omasal papillae contained such structures. Substance P-immunoreactive nerve cell bodies were numerous in the myenteric ganglia but were not detected in the omasal laminae. The present study failed to demonstrate intraepithelial nerve fibers in the omasum.

DISCUSSION

The current preparations demonstrated far more abundant nervous elements in the forestomach than the thin paraffin sections used in previous studies (Kitamura et al., 1986, 1987a). In addition, they demonstrated differences in innervation patterns between the calf and cow. The architecture of the nerve fiber network in the mucosal papillae appears to differ depending on the compartment of the forestomach examined (rumen, reticulum or omasum).

Mucosal nerve fibers including intraepithelial fibers have been demonstrated in the rumen and the reticulum of the lamb and kid utilizing methylene blue perfusion (Hill, 1959), in the reticulum of the sheep by cholinesterase activity (Comline and Message, 1965), and in the reticulum of the sheep and goat by the osmium-zinc-iodide method (Schenk-Sauber et al., 1985). The present study demonstrated for the first time intraepithelial nerve fibers having substance P-immunoreactivity in the rumen and reticulum of cattle. The free endings of intraepithelial nerve fibers are most likely to be sensory. Substance P is often contained in sensory nerve fibers associated with the gut (Sharkey et al., 1984; Furness et al., 1988; Bornstein et al., 1989; Costa et al., 1991). Substance P-immunoreactive intraepithelial nerve fibers in the forestomach are presumed to receive chemical and/or mechanical stimuli such as volatile fatty acids and/or distention from within the lumen of the forestomach.

The connective tissue core of the ruminal papillae contained a sparse number of fibers in the adult, while those of calf contained a few more fibers. There are some similarities in the distribution of nerve fibers in the ruminal papillae of the lamb and kid reported by Hill (1959) with those of the calf, though intraepithelial nerve fibers were more abundant in the lamb and kid. Although peculiar condensations of nerve fibers were not seen in the rumen, mucosal nerve fibers and ganglia were more abundant in the atrium than in the other parts of the rumen of both the calf and cow. Nerve fibers were very sparse in the rumen of the cow as compared to that of the calf. They were fewer in number in comparison with the reticulum and omasum also. We have the impression that the ruminal papillae of the cow contain too large a mesh of the network to demonstrate the entire picture of their framework by the present method.

Leek (1972) described the occurrence of silver-impregnated nerve fibers in the mucosa of the sheep reticulum and proposed a sensory role. We have demonstrated numerous substance P-immunoreactive nerve fibers in the tips of the reticular papillae of both the calf and cow. Sensory nerves in the intestinal mucosa may contain substance P (Sharkey et al., 1984; Furness et al., 1988; Bornstein et al., 1989; Costa et al., 1991). The topography of the dense areas of substance P-immunoreactive nerve fibers in the reticular mucosa suggests that these fibers are sensory in nature rather than secreto-motor. This concept is further supported by the fact that the distribution of the dense areas of immunoreactive nerve fibers is in accordance with the distribution of sensory receptors in the reticular mucosa as defined by physiological experiments (Leek, 1969; Leek and Harding, 1975).

Nerve fiber distribution in the mucosa of the lips of the reticular groove was sparse, even in the calf, although abundant substance P-immunoreactive nerves were present in the smooth muscle layer of the lips. Papillae on the floor of the groove showed a peculiar network of substance P-immunoreactive fibers. The architecture of this network appeared different from that observed in the reticular papillae. The reticulo-omasal orifice occupies an important position and is assumed to monitor ingesta passing through it (Titchen, 1962). For a more thorough understanding of this region, it will be important to gather more extensive morphological and physiologi-
nal data on the mucosal papillae around the reticulooomasal orifice, including the floor of the reticular groove and the initial part of the omasum.

A peculiar arrangement of glomerulus-like condensations of substance P-immunoreactive nerve fibers was found in the core of the omasal papillae of the calf. Similar structures immunoreactive for enkephalin derivatives were reported in the calf (Kitamura et al., 1987b). These nervous structures may respond to luminal stimuli during nursing. In his review, Bost (1970) assumed that omasum also has mucosal receptors. However, the exact location of these receptors has not been described. Such mucosal receptors may be represented by abundant nerve fibers revealed in the omasal papillae. A network of the mucosal nerve fibers was observed in the current study to be positioned immediately beneath the epithelium of omasal papillae of the cow rather than forming a condensed fiber network in the core of the omasal papillae. The sensory mechanism of the omasal mucosa may differ between the calf and the cow.

Mucosal nerve cell bodies immunoreactive for substance P were demonstrated in the rumen of the calf and in the reticulum of both the calf and cow. Many of them had a smooth surfaced unipolar appearance, sometimes exhibiting a long process directed toward the papillae. It is thought that a single mucosal ganglion innervates two or more papillae in the rumen as well as in the reticulum. However, mucosal/submucosal ganglia were not observed in the rumen of the cow or in the omasal laminae of either the calf or cow. It is possible that mucosal/submucosal ganglia are present in these regions, but they are too few in number to be demonstrated by current methods. These ganglion cells may cover a larger area than could be expected. Alternatively, it may be that large areas actually lack direct innervation from the mucosal/submucosal ganglia. Substance P-containing sensory fibers in the mucosa may be both intrinsic and extrinsic to the gut wall (Sharkey et al., 1984; Bornstein et al., 1989). Sensory nerve fibers in the mucosa of the ruminant forestomach may originate from a mucosal/submucosal ganglion or myenteric ganglion and also claim an extrinsic origin from the vagal fibers.

Results from the present study demonstrated an uneven distribution and density of mucosal nerves with substance P-immunoreactivity in the bovine forestomach. In addition, there were marked differences between nervous elements in the calf and the cow. Although the decreased density of mucosal nerves in the rumen of the cow may be attributable to the progressive development of the rumen, this does not explain why the immunoreactive nerve fibers in the omasal mucosa vary not only in the density but also in the pattern of distribution. The significance of the omasum in digestion and/or its importance in the regulatory mechanism of the bovine stomach remains unknown. The present immunohistochemical study serves to clarify the mucosal innervation of the omasum as well as the rumen and the reticulum and provides additional data concerning mucosal receptors in the bovine forestomach. It is hoped that, by gathering such data, the function of the ruminant forestomach may be more clearly understood in the future.

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REFERENCES


---: The innervation of sheep forestomach papillae from which combined chemoreceptor and rapidly adapting mechanoreceptor responses are obtainable. J. Physiol. (Lond.) 227: 229-239 (1972).


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