Intraepithelial Nerve Fibers in the Nasal Mucosa of the Rat with Special Reference to the Localization of CGRP, VIP and Nitric Oxide (NO)

Sang Hag Lee, Toshihiko Iwanaga and Tsuneo Fujita

Department of Anatomy, Niigata University School of Medicine, Niigata, Japan

Received February 10, 1995

Summary. Previous studies have demonstrated that the epithelium of the respiratory portion of rat nasal mucosa is amply supplied by nerve fibers with immuno-reactivities for calcitonin gene-related peptide (CGRP) and substance P (SP), these fibers most likely acting as sensory mediators in the mucosa. The present study demonstrates that some intraepithelial fibers contain a VIP-immunoreactivity whose occurrence in these nerves has previously been neglected. The present study further aims to confirm the occurrence of NO-producing intraepithelial nerve fibers in the rat nasal mucosa and to examine its colocalization with CGRP and with VIP.

Double staining methods were used to evaluate the colocalization of NADPH-diaphorase reactivity and CGRP- or VIP-immunoreactivity. The reactivity for NADPH-diaphorase and that for CGRP coexisted in only a small part, if any, of the nerve fibers distributed at the basal portion of the epithelium. In the perpendicularly and obliquely oriented transepithelial nerve fibers, both reactivities were clearly demonstrated to be separated in different fibers.

VIP immunoreactivity was also present in a part of the intraepithelial nerve fibers of the nasal mucosa, and their entire population was shown to be positive for NADPH-diaphorase.

The NADPH-diaphorase-positive reaction was displayed in only a small population of neurons in the trigeminal ganglion, whereas it was seen in numerous neurons in sphenopalatine ganglion, being colocalized with VIP.

The nasal mucosa, especially the epithelium of the respiratory area, receives an abundant sensory innervation that may be involved in protective reflexes elicited by the inhalation of airway irritants. Immunohistochemical studies have shown that the intraepithelial nerves contain substance-P (SP) and calcitonin gene-related peptide (CGRP). These peptides, which are known to be signal peptides for primary sensory neurons, have been demonstrated to coexist in neurons of the trigeminal ganglion and nerve fibers distributed in the nasal mucosa (Lundblad et al., 1983; Udman et al., 1985; Lundberg and Högfelt, 1986; Stjärne et al., 1989; Finger et al., 1990). Further, neurokinin derivatives were reported to occur in these nerves (Lundblad et al., 1983).

In the nasal mucosa, CGRP-immunoreactive nerves have been shown to be distributed even more extensively than the SP-immunoreactive fibers (Lundberg and Högfelt, 1986). Hence, CGRP has been described as a major mediator of the trigeminal chemoreaction and is favored as the immunohistochemical marker for the sensory nerves of the nasal mucosa (Silverman and Kruger, 1989; Finger et al., 1990; Moller et al., 1993).

The nasal mucosa, especially the submucosal glands, has been known to be richly supplied with vasoactive intestinal polypeptide (VIP), the predominant peptide contained in parasympathetic nerves, but its occurrence in the intraepithelial nerves was denied by Lundberg and Högfelt (1986). Recent studies using NADPH-diaphorase histochemistry and nitric oxide synthase (NOS) immunohistochemistry have demonstrated that, besides occurring in the perivascular and periglandular nerve fibers originating from the sphenopalatine ganglion, NO is also present in the intraepithelial nerve fibers of the nasal mucosa. It has further been suggested that NO-containing intraepithelial nerve fibers originate from the trigeminal ganglia (Hanazawa et al., 1993; Lee et al., 1995). This assumption was based on the knowledge that no neuropeptide other than CGRP and SP was found in the intraepithelial nerves of the
nasal mucosa as described above. As the present study reveals the intraepithelial occurrence of VIP-containing nerves in rat nasal mucosa, it seems necessary to re-investigate the coexistence of NO with this neuropeptide, besides that with CGRP/SP. The localization of those peptides in question and of NO is examined also in the trigeminal and sphenopalatine ganglia.

**MATERIALS AND METHODS**

**Tissue preparations**

Ten adult male Wistar rats (180–220 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and perfused through the aorta with physiological saline, followed by 150 ml of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After perfusion fixation, the nasal septal mucosa, trigeminal and sphenopalatine ganglia were removed and immersed in the same fixative for an additional 24 h. After immersion in 30% sucrose solution, they were embedded in OCT compounds (Tissue Tek®, Miles Scientific, Naperville, USA) and quickly frozen in liquid nitrogen. The frozen tissue blocks were sectioned at a 15 μm thickness in a cryostat and air-dried prior to further staining.

**Combination of NADPH-diaphorase histochemistry with immunostaining for CGRP**

1) Immunohistochemistry (ABC method) after NADPH-diaphorase histochemistry

Sections were first incubated for NADPH-diaphorase for 30 min with 0.1 M phosphate buffer (pH 7.4) containing 0.4 mg/ml nitroblue tetrazolium (NBT), 0.2 mg/ml nicotinamide-adenine dinucleotide hydrogen phosphate (NADPH)-diaphorase (β-NADPH) and 3 μl/ml Triton X-100. Thereafter, they were immersed in 0.3% Triton X-100-containing 0.1 M phosphate buffered saline (PBS, pH 7.2) for 30 min and then incubated with a rabbit polyclonal CGRP II antibody (Peninsula Lab., California, USA) at a dilution of 1:4,000 over 24 h at room temperature. They were then washed in PBS and incubated with biotin-labeled anti-IgG for 1 h, followed by streptavidin-conjugated peroxidase for 1 h (Histofine, Nichirei, Tokyo, Japan). Antibody binding was visualized with 0.05M Tris-HCl buffer (pH 7.6) containing 1% H2O2 and 10 mg diaminobenzidine (Wako Chemicals, Japan). The stained sections were dehydrated in a graded ethanol series and mounted with Eukitt® (O. Kindler GmbH & Co., Germany).

This double staining method showed intense positive reactions for both NADPH-diaphorase and CGRP. However, it was difficult to judge the coexistence of both reaction products because the dark blue staining for NADPH-diaphorase tended to disturb the recognition of the brown products by the peroxidase reaction. We therefore designed the following two double staining methods, combining an immunofluorescence method for CGRP with the NADPH-diaphorase histochemistry.

2) Immunofluorescence staining after NADPH-diaphorase histochemistry

Sections were stained for NADPH-diaphorase histochemistry as mentioned above. After treatment with 0.3% Triton X-100-containing PBS they were processed to the immunofluorescence method for CGRP; the CGRP antiserum was used at a dilution of 1:600. The site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG (Dakopatts, Denmark) at a dilution of 1:30 and observed under a Leitz Ortholux equipped with a fluorescence vertical illuminator (Ploemopak).

3) NADPH-diaphorase histochemistry after immunofluorescence staining

Sections were pretreated with 0.3% Triton X-100-containing 0.1 M PBS for 1 h and incubated with a
normal goat serum for 30 min. After rinsing, sections were incubated with the CGRP antiserum for 6 h at room temperature. The site of the antigen-antibody reaction was then revealed by FITC-labeled goat anti-rabbit IgG (Dakopatts, Denmark) at a dilution of 1:30. Sections were mounted with glycerin-containing 0.1% p-phenylenediamine (Wako Chemical Co., Japan). Several areas of the nasal mucosa were photographed under a Leiz Ortholux, and the location was memorized. After coverslips were removed from the sections, the glass slides were washed in PBS and subsequently treated for NADPH-diaphorase as

Figs. 2 and 3. Double staining of NADPH-diaphorase (a) and CGRP (immunofluorescence) (b) in the nasal mucosa. After NADPH-diaphorase staining, the section was incubated with anti-CGRP serum. In Fig. 2, both reaction products are localized in different intraepithelial nerve fibers. Fig. 3 demonstrates a part of the tissue where the NADPH-diaphorase (a) and the CGRP immunofluorescence (b) occur in a basal intraepithelial nerve of similar structure and localization (arrow). It is difficult to decide whether both reactivities are colocalized in the same fibers or separated in different fibers running in a small bundle. ×1,600
mentioned above. They were dehydrated in a graded alcohol and mounted with Eukitt®. The same sites previously photographed for the immunofluorescence reaction were photographed again under a conventional microscope. The colocalization of NADPH-diaphorase reaction and the immunofluorescence for CGRP was evaluated by comparing the photographs.

**NADPH-diaphorase histochemistry after immunofluorescence staining for VIP**

Sections were pretreated with 0.3% triton X-100-containing 0.1 M PBS for 1 h and were incubated with a normal goat serum for 30 min. Afterwards, they were incubated with the VIP antiserum (ICN ImmunoBiologicals) diluted 1:800 for 6 h at room temperature; all further steps were performed by the above mentioned procedures.

**RESULTS**

**NO versus CGRP**

1) **ABC method for CGRP after NADPH-diaphorase histochemistry**

NADPH-diaphorase-positive nerve fibers were stained dark blue while CGRP-immunoreactive fibers showed brown peroxidase products (Fig. 1). This combined staining was reproducible and reliable. Both types of nerve fibers entered the epithelium to form a nerve plexus located at the base of the epithelium. Both types of fibers partly emerged into the epithelial layer to reach the free surface. The intraepithelial nerves were beaded in appearance, with fine segments interposed between swellings; sometimes only the varicose portions were stained as dots, while the fibrous segments were invisible (Fig. 1). Such
dotted fibers ascended the epithelium either obliquely or perpendicularly. No essential difference in course or morphology was found between the NADPH-diaphorase-positive fibers and CGRP-positive fibers, except that the latter were more numerous in the epithelium than the former.

When nerve fibers were closely bundled, it was often difficult to exclude the possible colocalization of both reactions, because the dark blue color for NADPH-diaphorase obscured the brown peroxidase reaction. Careful examination at high magnification made it possible to recognize their separate localization in many fibers. The separate occurrence of both reactivities was evident in the case of the transepithelial nerve fibers, as these seldom ran close to each other (Fig. 1).

Another NADPH-diaphorase reaction was seen in the cytoplasm of some populations of the epithelial cells which completely lacked CGRP immunoreactivity (Fig. 1).

2) Immunofluorescence reaction for CGRP after NADPH-diaphorase histochemistry

This method made it possible to examine the coexistence of NADPH-diaphorase and peroxidase products more easily than the first procedure. The two reactivities appeared separately in the transepithelial nerve fibers (Fig. 2a, b). At the basal level of the epithelium, however, a small number of NADPH-diaphorase-positive nerves seemed to contain the CGRP immunoreactivity (Fig. 3 a, b).

In the trigeminal ganglion, CGRP-immunoreactivity was present in a major population of small-sized neurons. The intensity of the immunoreactivity varied according to the cells; some cells were intensely stained, with the immunoreactivity homogeneously distributed within the cytoplasm. In less intensely immunoreactive cells, the fluorescence displayed a perinuclear ring or granular distribution throughout the cytoplasm. In addition, axonal fibers with a very intense immunoreactivity were distributed between the CGRP-immunoreactive perikarya (Fig. 4b).

NADPH-diaphorase-positive cell bodies were also recognized in the ganglion, but corresponded only to a small number of the CGRP-immunoreactive ones. They were mostly small in size, though some medium-sized and occasional large cells were included. The positive reaction for NADPH-diaphorase was generally weak and homogeneously distributed in the entire cytoplasm (Fig. 4a).

Comparison of two positive reactions in the trigeminal ganglion indicated that only a part of CGRP-
immunoreactive cells were positive for NADPH-diaphorase, whereas the vast majority of NADPH-diaphorase-positive cells contained CGRP-immunoreactivity. However, a small number of NADPH-diaphorase-positive cells were negative in the CGRP-immunoreactivity (Fig. 4a, b).

3) NADPH-diaphorase histochemistry after immunofluorescence for CGRP

This double staining clearly distinguished two types of nerve fibers which gave the same result as that of the methods described above.

NO versus VIP

The VIP-immunoreactive nerve fibers, as is known, were heavily distributed around blood vessels and the acini of the seromucous glands. Surprisingly, they were also found in the epithelial layer of the nasal mucosa and ran along the basement membrane of the nasal mucosa, sometimes extending to the epithelial surface (Fig. 5). Thus, the intraepithelial nerve fibers immunoreactive for VIP were similar in distribution pattern and morphology to the CGRP immunoreactive nerves, though more restricted in distribution.

Double staining showed that essentially the entire population of VIP nerves were positive for NADPH-diaphorase in the periglandular and perivascular areas. Furthermore, VIP also coexisted with NADPH-diaphorase in the intraepithelial nerve fibers (Fig. 6a, b).

In the sphenopalatine ganglion essentially all neurons contained both VIP and NADPH-diaphorase
DISCUSSION

Since the wide distribution of NO-producing nerves was revealed in various organs in the late 1980s, the coexistence of NO with other transmitters has been investigated. In their double staining studies, some researchers evaluated the possible effects of the NADPH-diaphorase reaction on the results in succeeding immunohistochemical procedures (LOESCH et al., 1993; CHRISTENSEN and FANG, 1994). They have pointed out two problems in their experimental results: Firstly, NADPH-diaphorase reaction product, the formazan, is so heavily colored that it disturbs the visualization of the peroxidase reaction; secondly, the reaction product may possibly prevent the antigen-antibody coupling after NADPH-diaphorase reaction. When NADPH-diaphorase reaction was combined with the ABC method for CGRP in the present study, we also noted that the heavy blue-colored formazan made it impossible to distinguish two reactions which might be colocalized in the same fibers. To solve this problem, we combined the NADPH-diaphorase reaction with an immunofluorescence method. This double staining method allowed more precise judgment than the previous, non-fluorescence double staining as concerned the local reaction of CGRP and NADPH-diaphorase in the intraepithelial nerves, especially in the base of the epithelium where nerve fibers were closely bundled. Regarding the second problem that the formazan may possibly inhibit the antigen-antibody reaction, this possibility may be excluded by the present finding that the alternative staining of immunofluorescence and NADPH-diaphorase histochemistry demonstrated the same results. Moreover, this possibility is also refuted by the demonstration of the exclusive coexistence of VIP and NO in the intraepithelial as well as periglandular and perivascular nerve fibers.

Several previous studies using the enzyme histochemistry for NADPH-diaphorase and immunohistochemistry for NOS have described the presence of NO nerve fibers in the nasal and tracheal respiratory epithelia which are believed to be innervated mainly by sensory fibers (FISCHER et al., 1993; HANAZAWA et al., 1993; LEE et al., 1995).

On the other hand, the rich existence of CGRP in the nasal mucosa has been demonstrated by radioimmunoassay and by immunohistochemistry in several species, including humans (SILVERMAN and KRUGER, 1989; STJÄRNE et al., 1989; FINGER et al., 1990; MOLLER et al., 1993). There is general agreement that unmyelinated sensory fibers in the peripheral organs usually contain CGRP as well as SP; the former was noted to be more extensively and intensely immunostained in sensory nerves in various tissues, including nasal mucosa (LUNDBERG and HÖKFELT, 1986).

Previous researchers noted that the distribution pattern of NO nerves in the nasal epithelium overlapped with that of CGRP-containing nerves, forming a basal plexus within the epithelium extending upwards, close to the luminal surface (FISCHER et al., 1993; HANAZAWA et al., 1993; LEE et al., 1995). Besides these findings, the presence of NADPH-diaphorase and NOS in the trigeminal ganglion suggested that NO-containing intraepithelial nerves might be sensory in nature.

In contrast to this once widely accepted assumption, the present study, using double staining techniques, has demonstrated that a majority of NADPH-diaphorase-positive nerve fibers in the nasal epithelium lack CGRP immunoreactivity. The intraepithelial NO nerve fibers essentially contain VIP. Although no previous studies were able to succeed in demonstrating the presence of VIP-immunoreactivity in the intraepithelial nerve fibers of the nasal mucosa (LUNDBERG and HÖKFELT, 1986), the present finding supports the distribution, though not very extensive, of parasympathetic nerve fibers in the epithelial layer of the nasal mucosa. Previous immunohistochemical studies revealed that several perikarya of the sphenopalatine ganglion contained CGRP and SP (STJÄRNE et al., 1989; SILVERMAN and KRUGER et al., 1989; HARDEBO et al., 1992). Therefore, it is likely that CGRP-containing NO nerve fibers in the nasal epithelium may be partly originated from the sphenopalatine ganglion. This suggestion is supported by data that some CGRP and SP-positive fibers remain after capsaicin treatment or electrolytic lesioning of the trigeminal ganglion (STJÄRNE et al., 1989; FINGER and BÖTTGER, 1993). Taken together, these results suggest that a major population of intraepithelial NO-containing nerve fibers are originated from the sphenopalatine ganglion, indicating a non-sensory and autonomic nature, and that a minority displaying the colocalization of both CGRP and NADPH-diaphorase may be derived from the trigeminal ganglion, indicating a sensory nature.

A report is available showing the presence of acetylcholinesterase-positive reaction in the intraepithelial nerves, which may suggest the presence of parasympathetic mediator in the nasal epithelium (SPIT et al., 1993). However, there is no confirmatory evidence concerning sympathetic and parasympathetic
mediators occurring in the intraepithelial nerve fibers of the nasal mucosa. Our conclusion is in agreement with a recent study showing that the CGRP and SP nerves represent only a subpopulation of the intraepithelial nerves, indicating the presence of nerves containing unknown mediators (LUNDBLAD et al., 1984). In order to determine the origin of the different types of fibers in the epithelium more precisely, additional research, e.g., denervation experiments, are needed. The present results seem to indicate that the VIP-containing and NO-positive nerves do represent the "unknown" type of nerve.

REFERENCES


