Neuropeptides (1997) **31** (2), 167–173

Bombesin-like peptide is present in duct cells in salivary glands: studies on normal and irradiated animals

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Summary Bombesin (BN) and its mammalian counterpart gastrin-releasing peptide act as neuroregulatory hormones and tissue-specific growth factors, and have been implicated as peripheral and central satiety-inducing agents. In the present study, the immunohistochemical expression of BN in submandibular, sublingual and parotid glands of rats was examined 10 days after 5 consecutive days with daily doses of 6–8 Gy irradiation. Radioimmunoassay (RIA) methods were also used. Immunoreactive granular structures were observed within duct cells of both controls and irradiated animals. In the parenchyma of irradiated animals, very few nerve fibres showing BN-like immunoreactivity were observed. The RIA analysis showed that the content of BN-like material significantly increased in submandibular and parotid glands in response to irradiation. The results suggest that mainly a non-neural form of BN is detected in the salivary glands in the immunohistochemical analysis. Thus, the immunohistochemical observations suggest that BN-like peptides may be present in the duct system, where they may be constituents of the saliva. The observations of an increase in BN content in response to irradiation are of interest as BN has mitogenic effects, may stimulate secretion and contributes to satiety.

INTRODUCTION

Bombesin (BN) is a tetradecapeptide initially purified from the skin of the frog *Bombina bombina*.[1,2] One mammalian homologue of bombesin is the 27-amino acid peptide, gastrin-releasing peptide (GRP), isolated from porcine stomach.[3] Immunoreactivity for BN or GRP has been detected in the nervous system[4] as well as in macrophages[5] and neuroendocrine cells.[6,7] BN/GRP acts as neurotransmitter, paracrine hormone and tissue-specific growth factor in normal and neoplastic tissues.[8–11] Furthermore, BN/GRP is a potent satiety agent, being implicated in the physiological regulation of food intake.[12]

We have previously demonstrated that irradiation leads to an increase in the immunohistochemical expression of BN-like peptide in the innervation in submandibular glands[13] and submucosal glands in the larynx[14] of the rat. These observations may be of great importance since BN, as discussed above, functions as a neuroregulatory hormone, a tissue-specific growth factor and a satiety agent. Therefore, in the present study, we wanted to extend our previous immunohistochemical observations of a changed expression of BN in the submandibular gland in response to irradiation by also applying biochemical analysis and examinations of salivary glands other than the submandibular gland. Thus, radioimmunoassay analysis was performed in parallel with immunohistochemistry in a new set of studies on the effects of irradiation on rat salivary glands. All three major salivary glands (submandibular, sublingual and parotid) were analysed.

Received 12 September 1996
Accepted 15 January 1997

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MATERIALS AND METHODS

Animals

Twenty-nine 9-week-old female Sprague-Dawley rats were used. They had free access to water and pellets. Nineteen animals were subjected to bilateral irradiation of the head and neck region, 10 served as controls. Specimens from 6 irradiated animals and 5 controls were used for the

radioimmunoassay (RIA) analysis. Specimens from 13 and 5 rats, respectively, were used for the immunohistochemical studies. The protocol had been approved by the local ethical committee at the University.

Irradiation procedure

Irradiation was given with a medical linear accelerator, 6 MV, 6–8 Gy daily for 5 consecutive days, total dose 30–40 Gy. Total dose was 30 Gy for 9 of the animals, whereas 5 were treated with 35 Gy and 40 Gy, respectively. Specimens used for the RIA analysis were from animals that were treated with 30 Gy. During the irradiation, the rats were anaesthetized with Brietal (Methohexital), 0.2 mL in a tail vein, and fastened in a plastic mould holding them firmly in position. During irradiation, the rats were observed through a TV camera. The absolute dosimetry was checked with an ionization chamber in a rat-like phantom and all scattering materials in the field were kept constant.[15] The control rats were anaesthetized and, except for not being irradiated, were treated similarly to the experimental animals.

Immunohistochemistry

Tissue sampling and sectioning

Ten days after the last irradiation, all rats were anaesthetized with Mebumal (sodium pentobarbital 40 mg/kg i.p.). The submandibular, sublingual and parotid glands were dissected out and fixed by formaldehyde in 0.1 M phosphate buffer, pH 7.0. Thereafter the specimens were thoroughly washed in Tyrode's solution, containing 10% sucrose, at 4°C overnight and mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL). Submandibular, sublingual and parotid glands of controls were usually mounted together with corresponding glands of irradiated animals on the cardboard.

As a reference, the lumbar spinal cord of normal rats was dissected out and processed for immunohistochemistry (formaldehyde fixation). Spinal cord segments were mounted in OCT embedding medium as described above for the salivary gland specimens.

Series of 8–10 μm thick sections were cut using a cryostat. The sections were mounted on slides precoated with chrome-alun gelatin, dried and processed for immunofluorescence or stained for demonstration of activity of NADH-tetrazolium reductase (NADH-TR).[16] The NADH-TR staining served as a reference for tissue morphology.

Staining procedures

Sections were incubated for 30 min in a 1% solution of detergent Triton X-100 in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, rinsed three times for 5 min each in PBS, and incubated in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. The sections were then incubated with the primary antibody, diluted in PBS with BSA, in a humid environment. Incubation was performed for 60 min at 37°C. After incubation with specific antiserum and after three 10 min washes in PBS, the sections were immersed in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG diluted 1:40, for 30 min at 37°C in a moist chamber, washed in three changes of PBS, mounted in glycerol:PBS (1:1) and examined under a photomicroscope equipped with epifluorescence optics. For details, see Forsgren and Söderberg.[17]

Antibodies and preabsorption tests

The BN/GRP antibody used was raised in rabbits using synthetic bombesin conjugated to bovine thyroglobulin with glutaraldehyde (Incstar Corporation, Stillwater, MN; code: 368, working dilution 1:100). The specificity of the antiserum was tested by incubation of sections with antiserum preabsorbed with 10–20 μg of synthetic BN (Sigma, USA), or 10–20 μg of synthetic substance P (Sigma), in 1 mL of antiserum.

Radioimmunoassay (RIA)

Preparation of samples

Ten days after the last irradiation, control and irradiated rats were anaesthetized with Mebumal as described above. The submandibular, sublingual and parotid glands were removed, weighed and rapidly frozen in liquid nitrogen, whereafter the samples were stored at −80°C. The samples were minced while frozen, and extracted in 2 mL 1 M acetic acid at 100°C for 15 min. After boiling, the samples were homogenized by vortexing with a spatula. After homogenization and centrifugation (3000 × *g* for 20 min), the supernatants were collected and lyophilized. The samples were reconstituted and diluted in 1% BSA-borate buffer and assayed for BN at two different dilutions.

RIA of BN

The concentrations of BN-like material in the extracts were determined with RIA by using ^{125}I kits, purchased from Incstar Corp. (Stillwater, MN). The RIAs were performed according to instructions from the company. The BN antibody was directed against synthetic BN, and has been tested by Incstar to show 50% cross-reactivity with porcine GRP, but only <0.002% cross-reactivity with other peptides tested (eledoisin, physalamein, neurotensin and substance P). The detection limit was 40 pg/mL. Recovery of exogeneously added BN into tissue samples using this homogenization and extraction procedure averaged

92 ± 3.1%. Intra- and interassay coefficients of variation were 6.5% and 10.5%, respectively.

RESULTS

Animal weight

Ten days after irradiation, the average weight of the treated animals was 127 ± 12 g whilst that of the controls was 250 ± 11 g. All animals weighed approximately 210 g at the start of the experiments.

Immunohistochemistry

Sections of the salivary glands were regularly processed with BN antiserum that had not been preabsorbed ('staining for BN') and with BN antiserum that was preabsorbed with substance P (SP). Preabsorption with BN was also performed.

Immunoreaction in the innervation of salivary glands

Immunolabelled nerve fibres were observed in association with the large ducts in all three salivary glands after staining for BN (Fig. 1A). These immunoreactions were observed at similar extents in controls and irradiated animals. After staining with BN antiserum preabsorbed with SP, no such fluorescence reactions were detected (Fig. 1B).

In the irradiated animals, very few immunoreactive nerve fibres were observed in the parenchyme, i.e. in association with small ducts and acini, after staining for BN. No fibres at all were observed in these locations in the controls after staining for BN. Also after staining for BN preabsorbed with SP, very few immunoreactive nerve fibres were seen in the parenchyme of irradiated animals.

Immunoreaction in duct cells

Immunoreactive small granular structures were observed within duct cells of all three glands after staining with the BN antiserum, the pattern being the same with and without preabsorption with SP. These granular structures were observed in both control (Figs 2A,B) and irradiated (Fig. 2C) animals. Sometimes, small immunoreactive granular structures were also seen in the lumina of the ducts in both types of animals (Fig. 2D). Only occasionally were such structures observed within acini, and no such structures at all occurred in other structures present such as blood vessel walls and connective tissue spaces.

Different doses

The patterns of immunoreactions described above for irradiated animals were similar irrespective of total dose administered (30, 35, 40 Gy).

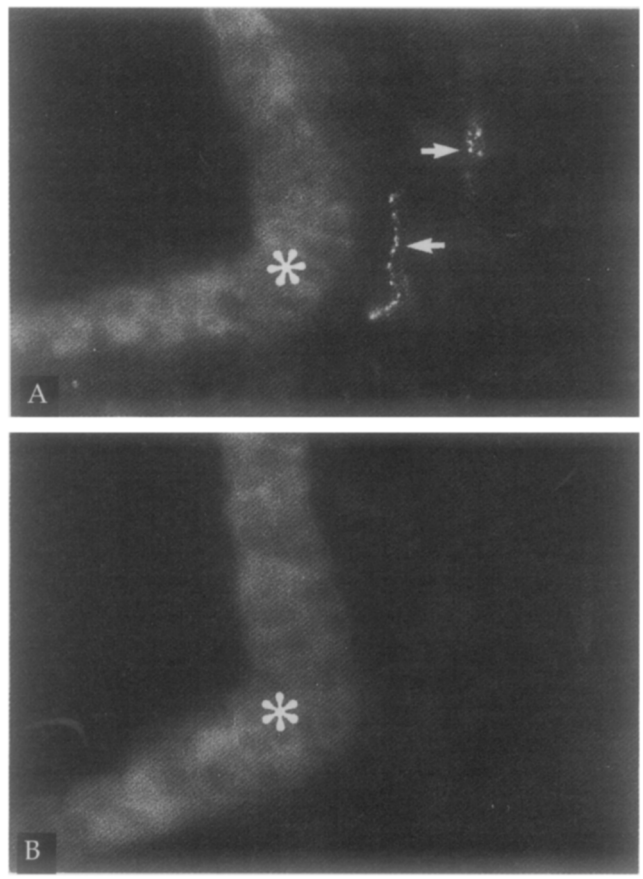


Fig. 1 Sections of a part of a large duct and adjacent tissue of a sublingual gland. Staining for BN (A) and staining for BN after preabsorption with SP (B). Asterisks mark corresponding regions in the duct. Immunoreactive varicose profiles are observed in (A). In (B) no immunoreactive profiles are observed. (× 600)

Preabsorption with BN

Preabsorption of the BN antiserum with synthetic BN completely abolished both nerve fibre staining as well as granular stainings in the salivary glands.

Immunoreaction in spinal cord

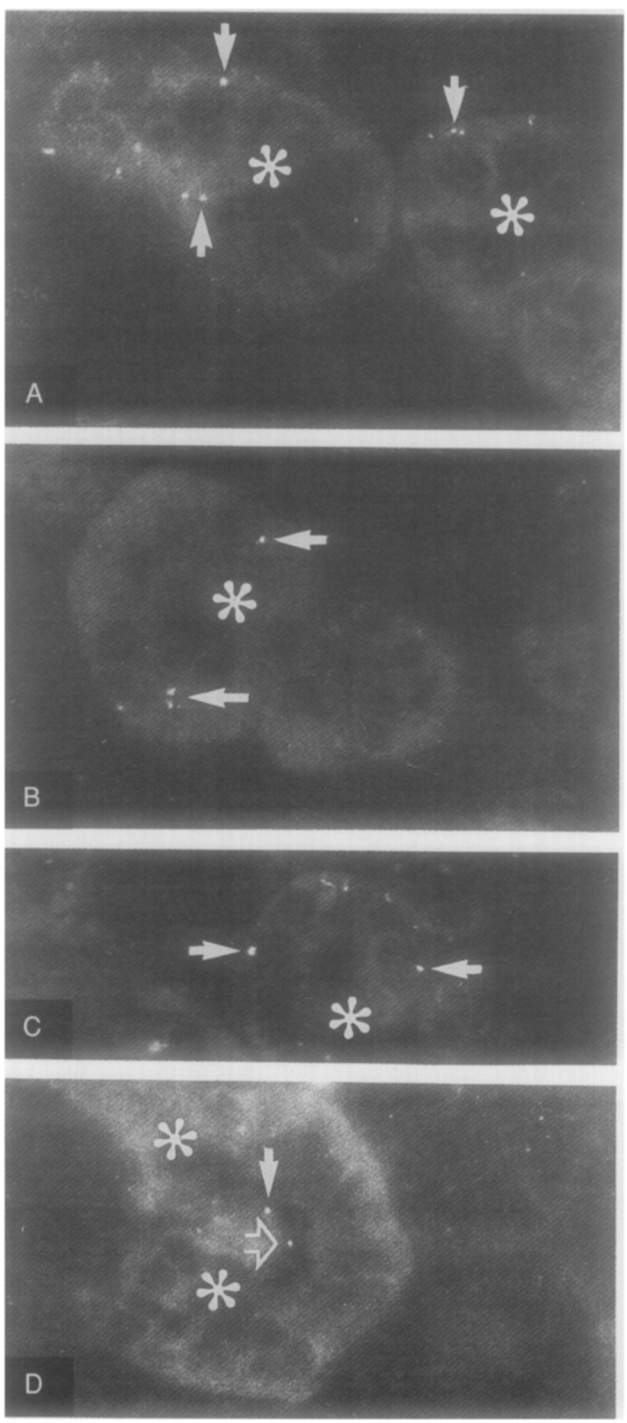
As an additional control of the BN antibody, staining of rat spinal cord was performed. Varicose nerve fibres were seen in the dorsal horn after staining for BN. Furthermore, varicose nerve fibres were also seen in this region after staining with BN antiserum that was preabsorbed with SP (not illustrated). The immunoreaction pattern resembled that seen by others in this region by use of other BN antisera.[18] Preabsorption with BN gave no specific reaction in these specimens.

Radioimmunoassay

The RIA analysis demonstrated an increase in BN-like content in submandibular and parotid glands 10 days after irradiation as compared to controls (Table 1 and Fig. 3).

DISCUSSION

The present study shows that BN-like immunoreaction is not only detectable in nerve fibres but also in duct cells in salivary glands. Furthermore, the content of BN-like peptide in the submandibular and parotid glands, as seen by RIA, was found to increase in response to irradiation. The weight of the animals dramatically decreased.



Unexpectedly, BN-LI was detected as fine granular structures in duct cells. Such an immunoreaction was not detected in our previous BN studies on the submandibular gland by use of another BN antibody.[13,19] To our knowledge, BN-LI has never previously been detected in duct cells, nor in other types of parenchymal cells in salivary glands, the BN immunoreaction in salivary glands being restricted to nerve fibres and ganglionic cells.[13] The most probable explanation is that the BN antibody used in the present study identifies only to a small extent a nerve-related BN-like peptide but identifies to a high degree another variant of BN-like peptide that is present in duct cells in salivary glands. In accordance with such a proposal, it has been suggested that BN-like peptides exist in at least two forms, one form considered to be produced in endocrine tissue and the other in neural tissue.[20,21] It is also known that BN antisera may contain antibodies directed towards the C-terminal decapeptide that BN-like peptides share with SP and its related peptides.[21] However, the peptide detected in the duct cells, as well as that in the innervation in the parenchyme, is apparently not SP-like, as preabsorption of the BN antiserum with SP did not change the pattern of immunoreaction in these parts. On the other hand, the BN reaction in the nerve fibres associated with the large ducts was found to cross-react with SP.

Immunoreactive granular structures were not only detected in the duct cells but also sometimes in the lumina of the ducts. Whether the occurrence of immunoreactive structures in the lumina are due to artefactual rifts in the membranes of the duct cells, rendering entrance of the granular structures into the lumina, or display an immunoreaction of released peptide is unclear. In future studies, it should be clarified as to whether BN indeed is released from the duct cells, thus becoming a constituent of the saliva. In a previous in vitro study, another neuropeptide, vasoactive intestinal polypeptide (VIP), was immunohistochemically detected in cultured duct cells, from a human parotid gland adenocarcinoma gland tissue.[22] In that study, the duct cells were found to express VIP in combination with amylase. Of interest are also the observations of immunoreaction of still another neuropeptide,

Fig. 2 (A, B) Sections of submandibular glands of control animals. Staining for BN after preabsorption with SP (A), ordinary staining for BN (B). Asterisks indicate ducts. Immunoreactive granular structures are observed in the duct cells (arrows). (× 500). (C) Section of a submandibular gland of an irradiated animal, after staining for BN. Asterisk marks a duct. Small immunoreactive structures are observed in duct cells (arrows). (× 500). (D) Section of a submandibular gland of a control animal after staining for BN. Two small immunoreactive granular structures are observed, one of which being located in the duct lumen (open arrow). (× 500).

Table 1 The content of bombesin in salivary gland tissue analysed by RIA expressed as pg/mg tissue (mean ± SEM)

	Bombesin (pg/mg tissue)
Submandibular gland	
Control (n = 5)	0.59 ± 0.02
30 Gy (n = 6)	0.95 ± 0.07
Parotid gland	
Control (n = 4)	1.10 ± 0.11
30 Gy (n = 5)	1.67 ± 0.12
Sublingual gland	
Control (n = 5)	1.26 ± 0.05
30 Gy (n = 4)	1.38 ± 0.06

enkephalin, in the saliva of man.[23] If BN-LI stored in the duct cells is released into the lumen of the ducts and then becomes included in the saliva, the peptide can possibly have effects on the mucosa of the gastrointestinal tract. In fact, BN given orogastrically has significant effects in the gastrointestinal tract.[24] It has also previously been proposed that BN may have a trophic response in the pancreas of suckling rats,[25] as BN-LI immunoreactants are present in maternal milk.[26,27]

The BN antibody used in the RIA analysis has been tested for cross-reactivity with various peptides and has been found to show only <0.002% cross-reactivity with all peptides tested. The findings of an increase in BN content is interesting as BN acts as a mitogen for various cell types, such as 3T3 mouse fibroblasts,[28] human normal bronchial epithelial cells,[8] and small cell lung carcinoma (SCLC) cells.[29,30] It is also known that SCLC cells are stimulated to proliferate by exogenous BN/GRP.[29] The observations in the present study of an increase of BN-like peptide in submandibular and parotid glands in response to irradiation might imply that BN has mitogenic effects in this situation. Thus, during the recovery following radiation-induced tissue injury, the increase in BN-like peptide may be involved in the repair process, contributing to the cellular renewal and recovery following radiation-induced salivary gland damage. It can also not be excluded that BN stimulates secretion in the salivary glands. Whether it indeed has such effects is however unknown. What is known is that BN stimulates exocrine and endocrine pancreatic secretion[31,32] and gastrin and pepsinogen release.[33–35] The reason for the lack of significant increase of BN-like peptide in the sublingual gland is unclear.

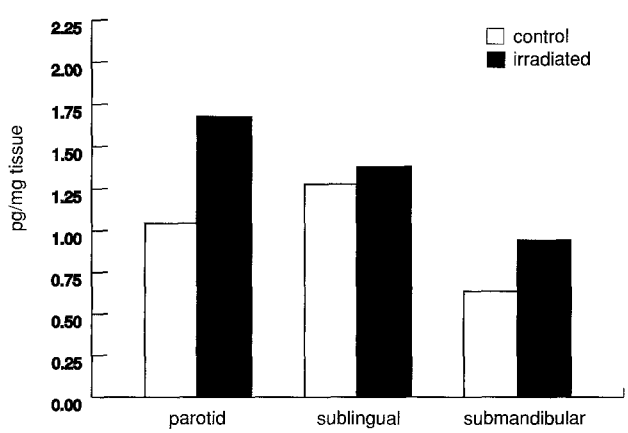


Fig. 3 Content of BN-like material in the major salivary glands of control animals (n = 5 for all three different glands) and after irradiation (n = 4 for parotid and sublingual glands and n = 6 for submandibular glands). Student's t-test resulted in the following P values: 0.012 for the parotid, 0.145 for the sublingual and 0.001 for the submandibular glands.

The treated animals decreased markedly in weight. Weight loss in this situation may be due to a number of factors such as altered food preference and lack of food intake related to mucositis and dysphagia. However, it is tentative to suggest that there might also be an association between the very pronounced decrease in body weight and the increase in BN content in the salivary glands. The reason for this speculation is the fact that BN contributes to satiety (see McCoy and Avery[36] for a review). Thus, it is known that both central and peripheral injections of BN inhibit food intake in rats.[37,38] As the observed immunoreactivity of BN in duct lumina might be indicative of secretion of BN from the duct cells and subsequent influence of the peptide, it is of interest to recall that there is a wide distribution of BN binding sites in the gastrointestinal tract of the rat.[39] The possibility of BN acting as a circulatory hormone must also be considered. Furthermore, it should be stressed that other neuropeptides, like various opioid peptides, can be involved in the feeding behaviour.[40]

In summary, this is the first report suggesting that BN/GRP is present in the duct cells of salivary glands. Thus, BN appears not only to be present in neuroendocrine cells, nervous structures and macrophages. The study also provides quantitative biochemical data suggesting that irradiation to head and neck region induces an increase in the content of BN/GRP-like peptide in submandibular and parotid glands. The increase of BN/GRP may be involved in the repair mechanisms and recovery of damaged cells following irradiation, since BN/GRP not only has neurotransmitter/neuromodulatory effects, but also is a growth factor.

ACKNOWLEDGEMENTS

We are grateful to Ms M. Wranding, U. Hedlund and L. Crenshaw for excellent technical services. The study was supported by grants from the Swedish Medical Research Council (10364), the Faculty of Medicine, Umeå University, the Lions Cancer Research Foundation,

Umeå, Lundbergs Forskingsstiftelse, Göteborg, and Gunnar, Arvid and Elisabeth Nilssons Stiftelse, Lund, Sweden.

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