Acta Derm Venereol 2004; 84: 428–432

Taylor & Francis healthsciences

INVESTIGATIVE REPORT

Galanin Expression in a Murine Model of Allergic Contact Dermatitis

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Galanin is a neuropeptide widely distributed in the nervous system. The expression of galanin was investigated in murine contact allergy using immunohistochemistry, radioimmunoassay and in situ hybridization. Female BALB/c mice were sensitized with oxazolone and 6 days later challenged on the dorsal surface of ears, while control mice received vehicle. After 24 h, one ear was processed for immunostaining using a biotinylated fluorescence technique, while the other ear was frozen and processed for radioimmunoassay or in situ hybridization. Galanin immunoreactive nerve fibres were more numerous ($p<0.01$) in the eczematous compared with control ears. Double-staining with antibody to the nerve fibre marker PGP (protein gene product) 9.5 revealed co-localization of PGP 9.5 and galanin in nerve fibres. Radioimmunoassay demonstrated a decrease ($p<0.04$) in galanin concentration in eczematous compared with control ears. Our results suggest a role for galanin in murine contact allergy. ***Key words: allergic contact dermatitis; mouse; neuropeptides; sensory nerves; immunohistochemistry; radioimmunoassay.***

(Accepted May 10, 2004.)

Acta Derm Venereol 2004; 84: 428–432.

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Galanin is a 29 (30 in human) amino acid peptide discovered in 1983 in extracts from the porcine jejunum (1). It is widely distributed in the nervous system, and it is also found in some neuroendocrine cells (2). In the rat central nervous system, galanin coexists with classical neurotransmitters (acetylcholine and noradrenaline) as well as with serotonin in a subpopulation of dorsal raphe neurons (3). Galanin has multiple biological effects and may alter the release of several neurotransmitters. Moreover, it may be involved in other neuronal functions such as learning, memory and nociception, as well as inflammation (4, 5).

In rat dorsal root ganglia only few galanin-positive neurons can be seen under normal circumstances (6, 7), but there is a strong upregulation in response to peripheral axotomy both in mouse (8, 9) and rat (10). Galanin is upregulated in dorsal horn neurons after peripheral inflammation (11), and has also been shown to be involved in the development of inflammatory arthritis in the rat (12, 13). In addition, galanin concentration is increased in chemically induced ileitis (14). It has also been reported that carrageenan-induced inflammation in rat skin causes a marked increase in galanin mRNA levels in cells in the epidermis, as well as in the dermis; however, no galanin immunoreactive nerve fibres were seen (15). Baumann et al. (16) showed presence of galanin immunoreactive nerve fibres in lamina propria, and galanin mRNA in epithelial keratinocytes of rat molar gingiva.

Allergic contact dermatitis is a common disorder caused by specific inflammatory reactions. It is a delayed-type hypersensitivity reaction consisting of an induction (sensitization) phase and an elicitation (challenge) phase (17). During sensitization the skin is exposed to a contact allergen (mostly a hapten), which induces a cascade of immune responses including the generation of memory T cells. Upon subsequent challenge, the memory T cells travel to the site of hapten contact and produce the characteristic skin reaction (17).

A possible role of galanin in contact hypersensitivity has not yet been investigated. We therefore studied the expression of galanin in induced allergic contact dermatitis in mice.

MATERIALS AND METHODS

Animals

Sixteen female BALB/c mice (Charles River, Uppsala, Sweden), aged 8–10 weeks, were used. For induction of contact allergy, a modification of the procedure described by Goebeler et al. (18) was applied. Briefly, eight mice were sensitized on 2 consecutive days by applying 100 µl of 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich, Sweden) dissolved in olive oil/acetone (1:4) on the shaved back. They were challenged 6 days later by painting 50 µl of 0.1% oxazolone dissolved in arachis oil/acetone (1:4) on the dorsal surface of both ears. The increase in ear thickness was measured with a spring-loaded micrometer (Kroeplin, Schluchtern, Germany) before and 24 h after

© 2004 Taylor & Francis. ISSN 0001-5555
DOI: 10.1080/00015550410017290

challenge and expressed in units of 10^{-2} mm. Control mice ($n=8$) were painted with the vehicle and kept in separate cages.

Ethical approval was obtained from the local animal ethics committee.

Mice in both groups were sacrificed 24 h after challenge, and one ear was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.0, for 4 h at 4°C, then rinsed with 0.1 M phosphate buffer containing 10% sucrose for at least 24 h, and sectioned on a Dittes cryostat (Heidelberg, Germany) (section thickness 14 μm). The sections were then processed for immunohistochemistry. The other ear was directly frozen on dry ice and stored at −70°C for radioimmunoassay (RIA) or in situ hybridization. In order to include areas with different degrees of inflammation, sections were cut at approximately 100-μm intervals and mounted on the slides. Nerve fibres were counted using a coded method. Three sections per mouse ear were counted, and the mean values were calculated.

Immunohistochemistry

Sections were incubated with rabbit anti-galanin (porcine) antibody (Bachem, St Helens, UK) at a dilution of 1:10,000 overnight at 4°C in a humid atmosphere. The following day, sections were rinsed in PBS for 10 min with three changes and were then incubated with a biotinylated goat anti-rabbit secondary antibody (dilution 1:200; Vector, Burlingame, CA, USA) for 40 min at room temperature. The primary antibody was visualized by incubating the sections with the fluorochrome Cy2 (dilution 1:2000; Amersham Pharmacia Biotech, Uppsala, Sweden). Finally sections were mounted with glycerol/PBS (10:1) containing 0.1% para-phenylenediamine, and coverslipped. For controls the primary antibody was omitted, or the antiserum was pre-adsorbed with galanin 10^{-4}, 10^{-5} or 10^{-6} mol/l.

In the double-staining experiment the sections were incubated with the polyclonal rabbit anti-galanin antibody and biotinylated goat anti-rabbit secondary antibody, followed by the incubation of a polyclonal guinea pig anti-PGP (protein gene product) 9.5 antibody (dilution 1:2000; Neuromics, Minneapolis, MN, USA) and a biotinylated goat anti-guinea pig secondary antibody (dilution 1:200; Vector). The staining was visualized using fluorochromes Cy2 (dilution 1:2000) and Texas red (dilution 1:800, Vector), respectively. The sections were examined with a Nikon Eclipse E 800 (Yokohama, Japan) microscope equipped for epifluorescence. Cy2 fluorescent structures were visualized with a B-1E filter cube (excitation at 465–495 nm), while Texas red fluorescent elements were seen with a G-1B filter cube (excitation at 540–580 nm). Coloured images were generated using a digital video camera system (Nikon DXM 1200, Yokohama, Japan) attached to the fluorescence microscope and connected to a PC.

Radioimmunoassay

For RIA the frozen ears were cut into small pieces and boiled for 10 min in 10 vol of 11 mol/l acetic acid. After homogenization, using a polytron and a Vortex mixer, the samples were centrifuged at 2500 *g* for 10 min. The supernatants were lyophilized and kept at −20°C until analysis. The precipitates were dissolved in 0.05 mol/l phosphate buffer, pH 7.4, before analysis (19).

The galanin concentration was determined using antiserum RatGala4 raised against conjugated synthetic rat galanin (19). This antiserum does not cross-react with substance P, neurokinin A, neurokinin B, neuropeptide K, neuropeptide Y, gastrin, pancreatic polypeptide, glucagon or neurotensin. HPLC-purified [^{125}I]-rat galanin was used as radioligand and rat galanin as standard. The detection limit of the assay was 5 pmol/l. Intra- and inter-assay coefficients of variation were 6% and 10%, respectively.

In situ hybridization

Treated and control ears were rapidly frozen and cut in a Dittes cryostat (as above) at 14 μm thickness. An oligonucleotide probe complementary to nucleotides 324–371 of rat galanin mRNA (20) (SGS/AB, Köping, Sweden) was purified through QIA quick spin columns (QIAGEN/Gmbh, Hilden, Germany). DTT was added to a final concentration of 10 mM. The specific activities obtained ranged between 0.6 and 1.8×10^9 cpm/μg oligonucleotide.

The tissues were hybridized according to published procedures (21). Briefly, the sections were covered with a hybridization buffer, placed in a humidified chamber and incubated for 16–18 h at 42°C. After being rinsed and air-dried, the sections were dipped in NTB2 nuclear track emulsion (Kodak), exposed, developed, fixed and mounted in glycerol-phosphate buffer. Some sections were subsequently stained with cresyl violet and mounted. Sections were analysed with a microscope equipped for bright-field and dark-field illumination (Microphot FX, Nikon, Japan).

Statistical analysis

Student's t-test and Mann-Whitney U test were used for statistical analysis.

RESULTS

Topical application of oxazolone led to a visible erythema and significant oedema: the mean ± SD thickness was 0.29 ± 0.03 mm in eczematous (post-challenge) versus 0.22 ± 0.01 mm ($p<0.001$) in control (pre-challenge) ears.

Immunohistochemistry showed galanin-positive fibres in the dermis running parallel as well as perpendicular to the epidermis (Fig. 1a). Nerve bundles were also seen in the dermis and double-staining revealed the co-expression of PGP 9.5 with galanin-like immunoreactivity in transversely cut fibres in small nerve bundles (Fig. 1b and c). Galanin immunoreactivity was abolished by blocking with peptide at 10^{-6} mol/l (Fig. 1d).

The number of galanin immunoreactive fibres was increased ($p<0.01$) in the eczematous tissue (median 8.0, quartile deviation 4.5 fibres per section) compared with control tissue (2.5, quartile difference 2.5 fibres per section) (Fig. 2).

RIA analysis revealed a decrease ($p<0.04$) in galanin concentration (median 1.7, quartile deviation 1.0 pmol/g) in the eczematous ears compared with control ears (2.4, quartile deviation 1.3 pmol/g) (Fig. 3).

In situ hybridization of the ear sections did not reveal a positive signal for galanin mRNA in the mouse ears (data not shown).

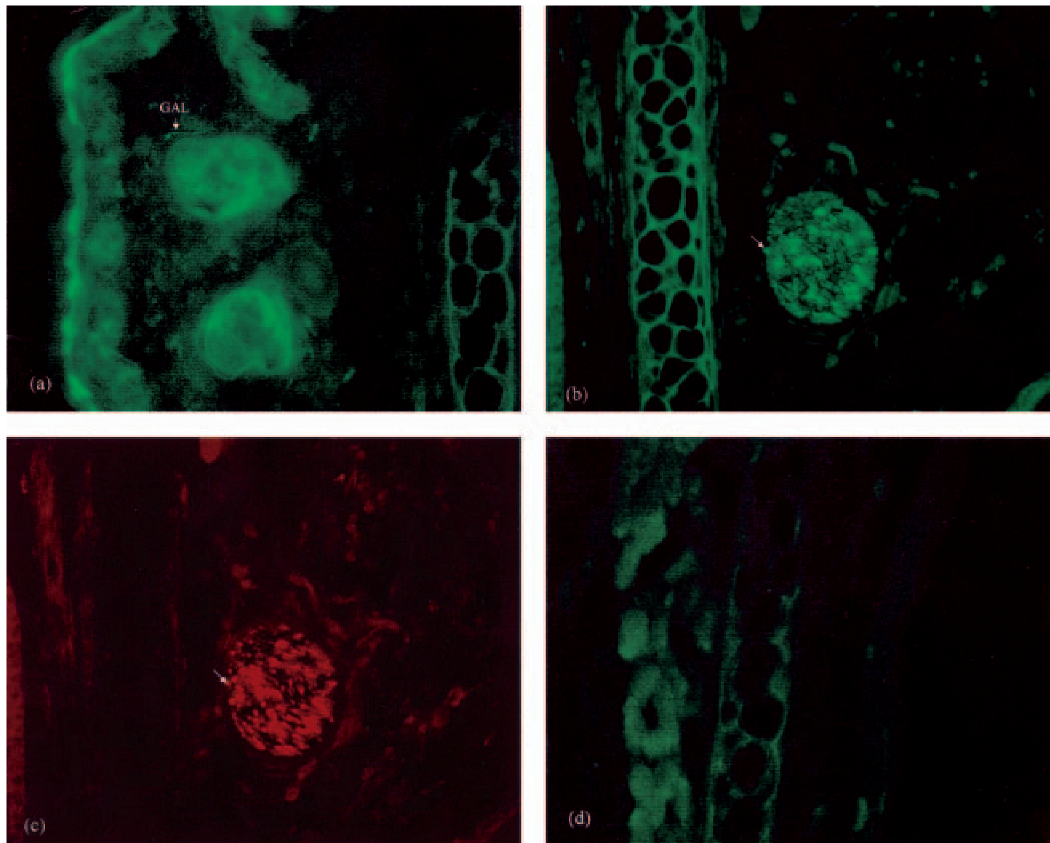

Fig. 1. Galanin expression in a nerve fibre (arrow in a, ×400) and in a nerve (b, c, ×200) in allergic contact dermatitis skin. Co-localization of galanin (b, Cy2) and PGP 9.5 (c, Texas red) in a nerve fibre (arrow). Note that some PGP 9.5 positive nerve fibres are negative for galanin (b, c). After pre-adsorption with an excess of galanin peptide no fluorescent fibres can be seen (d, ×400).

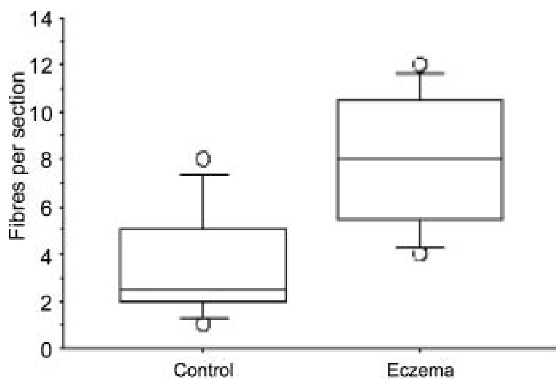

Fig. 2. Quantification of galanin immunoreactive nerve fibres in control and eczematous ears ($n=8$), shown in box plot (median, lower and upper quartile). Significance of difference $p<0.01$.

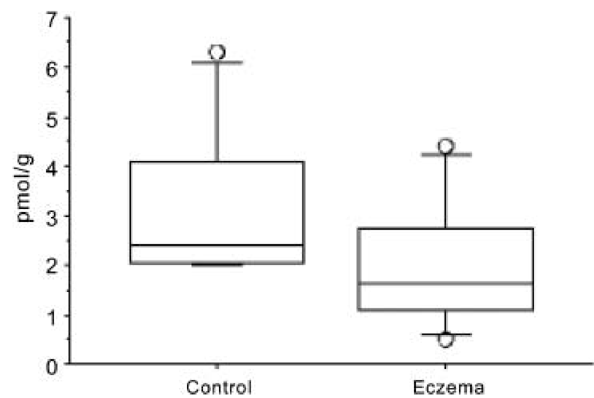

Fig. 3. Concentration of galanin in control and eczematous ears ($n=8$), shown in box plot (median, lower and upper quartile). Significance of difference $p<0.04$.

DISCUSSION

The present study reports on expression of galanin in the skin in a murine model of allergic contact dermatitis. An increase in galanin-positive fibres is observed in the dermis of eczematous mouse ears compared with control ears. The distribution of these fibres suggests that they are of sensory origin. The increase in their number indicates a possible increase in the intraneuronal galanin levels, a 'filling up' process, in a population of sensory neurons in response to a contact eczematous reaction. This in turn could be due to increased synthesis or decreased release. However, the possibility of sprouting might also be considered. In fact we have observed a significant increase in the density of growth associated protein-43 (a marker for axonal growth) positive nerve fibres in such eczematous compared with control ears at the same time point, 24 h (unpublished results).

However, our RIA analysis demonstrated statistically significant lower galanin concentrations in the eczematous skin as compared with control skin. The latter result is in line with previous findings by Ek & Theodorsson (22) of lower concentrations of sensory neuropeptides (tachykinins and calcitonin gene-related peptide, CGRP) in murine contact eczematous ears in contrast to control ears. Similar findings have also been demonstrated in a RIA analysis of substance P concentration in atopic eczematous skin lesions (23).

The reason(s) for the disparity between the immunohistochemical and RIA results is unclear. It is possible that an increased peripheral release of peptide followed by rapid breakdown by endopeptidases and mast cell proteases could explain the lower concentration in the eczematous ears, in spite of a higher number of galanin-positive nerve fibres. Slow axonal transport from the site of synthesis of this peptide could also lead to lower concentration in the eczematous ears. In addition, a decreased synthesis by non-neuronal cells, not detectable by immunohistochemistry in the present study, might contribute.

When applying in situ hybridization to our specimens no galanin mRNA-labelled cells could be detected. This is in contrast to a previous study by Ji et al. (15) on inflamed rat skin after carageenan-treatment, where galanin mRNA was observed in cells in dermis and epidermis. Maybe our technique was not sensitive enough to detect galanin mRNA in non-neuronal cells in the present study. Alternatively the two ways of inducing inflammation may cause a different response.

An effect of galanin on T cells was not investigated here; however, it has been reported that galanin may have an antiproliferative and pro-apoptotic effect on immature thymocytes (24).

Immunohistochemistry, in situ hybridization, RIA and receptor binding studies have revealed the distribution of galanin and galanin receptors in dorsal horn neurons and ganglia. In the latter, galanin was found to often coexist with substance P and CGRP in sensory neurons (6, 25, 26). Substance P and CGRP peptides are implicated in the pathogenesis of allergic contact dermatitis, having a vasodilator effect on blood vessels, enhancing plasma extravasation, as well as inducing chemotaxis of specific leukocyte subpopulations (18). Galanin, on the other hand has been shown to inhibit plasma extravasation (27), perhaps due to interaction with substance P and CGRP at pre- or postsynaptic levels. Thus a lower concentration of galanin, as shown here in the inflamed ears, might contribute to the pathophysiology of allergic contact dermatitis, through enhancement of plasma extravasation.

In conclusion, the presence of galanin in dermal nerve fibres, and the decreased content of this neuropeptide in inflammatory skin indicate an involvement of galanin in the pathophysiology of allergic contact dermatitis in mice.

ACKNOWLEDGEMENTS

This study was supported by grants from the Ekhaga Foundation, Konsul Th Berghs Foundation, the Welander/Finsen Foundation, Karolinska Institutet and the Swedish Research Council.

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