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Calcitonin gene-related peptide (CGRP) immunoreactivity in the neuroendocrine Merkel cells and nerve fibres of pig and human skin

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Summary. The presence of calcitonin gene-related peptide (CGRP) in the skin of pig snout and human fingertip was investigated using immunohistochemical techniques. CGRP immunoreactivity was found in Merkel cells and nerve fibres of both species. In pig snout skin, Merkel cells containing CGRP were seen forming clusters at the tips of rete ridge epidermis and in the external root sheath of sinus hair follicles (vibrissae). Human Merkel cells immunostained for CGRP were found isolated or forming small groups in the basal layer of glandular epidermal ridges. In all cases, immunoreactivity was more intense on the side of the Merkel cell facing the associated nerve terminal (which was never positive for CGRP). This part of the Merkel cell has the greatest density of dense-cored granules, suggesting that CGRP must be stored in these granules. Nerve bundles containing CGRP-immunoreactive fibres were found at dermal and hypodermal level, and blood vessels were often surrounded by CGRP nerve fibres. In pig snout skin some nerve fibres containing CGRP penetrated the epidermis and terminated as free endings, and in the human fingertip a small number of CGRP-immunoreactive nerve fibres were seen in Meissner's corpuscles.

Introduction

Calcitonin gene related peptide (CGRP) is a peptide of 37 aminoacids that is encoded by the calcitonin gene but is chemically unrelated to calcitonin; it was first identified by Amara et al. (1982) and Rosenfeld et al. (1983). Immunohistochemical studies have shown that CGRP is widely distributed in the central and peripheral nervous systems and in the endocrine or neuroendocrine diffuse systems (Rosenfeld et al. 1983; Gibson et al. 1984; Kawai et al. 1985; Rodrigo et al. 1985a, b; Terenghi et al. 1985; Cadieux et al. 1986; Kameda 1987; Lauweryns and Van Ranst 1987; Kuramoto et al. 1987; Scheuermann et al. 1987).

Merkel cells are cutaneous neuroendocrine cells characterized by the presence of electron-dense granules in the region nearest the nerve endings usually associated with these cells. Immunohistochemical studies have shown that Merkel cells express cytokeratin polypeptides (Saurat et al. 1984; Moll et al. 1984; Ortonne and Darmon 1985; Ness et al. 1987), desmosomal proteins (Ortonne and Darmon 1985), Pr h antigen (Saurat et al. 1983) and neuroendocrine markers such as neuron specific enolase (Gu et al. 1983; Warner et al. 1983; Zaccone 1986), chromogranin A (Ness et al. 1987) or synaptophysin (Ortonne et al. 1988; Garcia-Caballero et al. 1989). In Merkel cells were detected met-enkephalin in rodents (Hartschuh et al. 1979, 1980; Warner et al. 1983), vasoactive intestinal polypeptide in mammals, including man (Hartschuh et al. 1983, 1984), and serotonin in a fish, the conger-eel (*Conger conger*) (Zaccone 1986). The authors suggest that these substances were presumably stored in the dense-cored granules of Merkel cells.

CGRP has recently been found in the Merkel cells of cats (Alvarez et al. 1988b). The aim of the present study was to investigate the occurrence of CGRP in the Merkel cells and cutaneous nerve fibres of pig and man.

Materials and methods

Tissue specimens from four pigs (snout) and three humans (fingertips) were immersion-fixed in Bouin's fluid for 2 h, dehydrated with a graded ethanol series and embedded in paraffin. Sections 5 μm thick were cut perpendicularly to the cutaneous surface, mounted on glass slides coated with chromealum-gelatin and dried overnight at 37° C. After dewaxing and rehydratation, the sections were surrounded by Sigmacote (Sigma, St. Louis, USA) and rinsed with 0.01 *M* phosphate buffered saline of pH 7.4 (PBS). The streptavidin-biotin bridge technique was used for immunohistochemistry (Bonnard et al. 1984). Following blocking steps, the sections were consecutively incubated in: *1)* rabbit antiserum to CGRP (Amersham, Buckinghamshire, England) at a dilution of 1:600, (overnight at 4° C); *2)* biotinylated antibody to rabbit (Biomakor, Rehovot, Israel) at a dilution of 1:20 (for 30 min at room temperature); *3)* streptavidin-peroxidase (Biomakor, Rehovot, Israel) at a dilution of 1:20 (for 30 min at room temperature); *4)* 3,3′ diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, USA) at a dilution of 0.06% with 0.003% H_2O_2 (for 10 min at room temperature).

Between steps, the sections were rinsed with PBS (2 × 5 min). After step 4, they were rinsed with distilled water, dehydrated, cleared and mounted.

Specificity was checked using non-immune rabbit serum in place of the primary antiserum or with PBS in place of one of the other incubation steps. Preabsorption control was performed by preincubation of CGRP antiserum with CGRP (Bachem, Torrance, CA, USA) at a dilution of 20 μg/ml of diluted antiserum (overnight at 4° C).

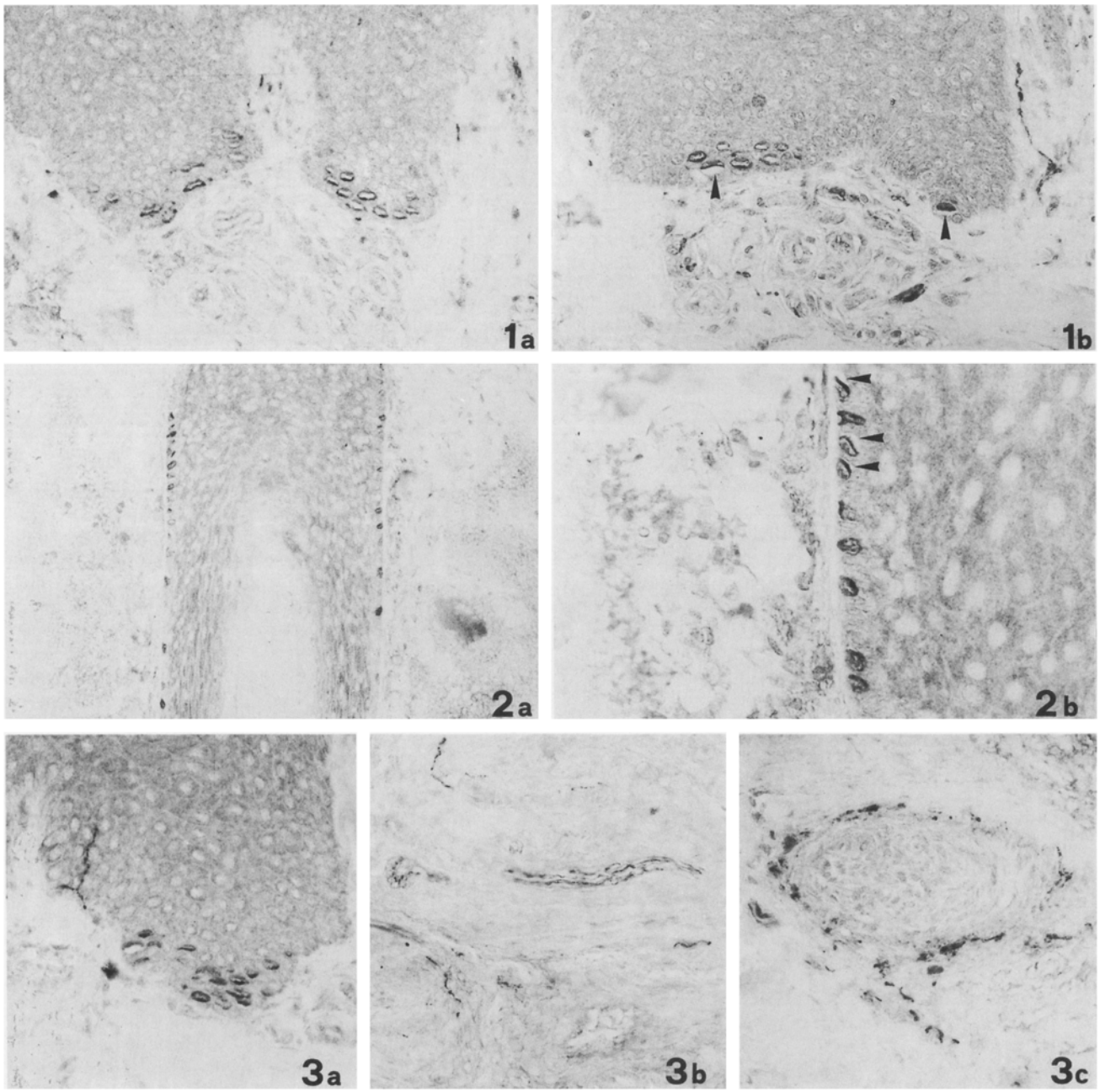


Fig. 1a–b. Pig snout, epidermis. **a** Groups of Merkel cells immunostained for CGRP located at the base of epidermal ridges (250 ×). **b** Epidermal Merkel cells exhibit most immunoreactivity on the basal side facing nerve endings. The latter (*arrowheads*) appear as clear discs under Merkel cells. CGRP-containing nerve fibres are seen below the ridge or running into the dermal papilla (300 ×)

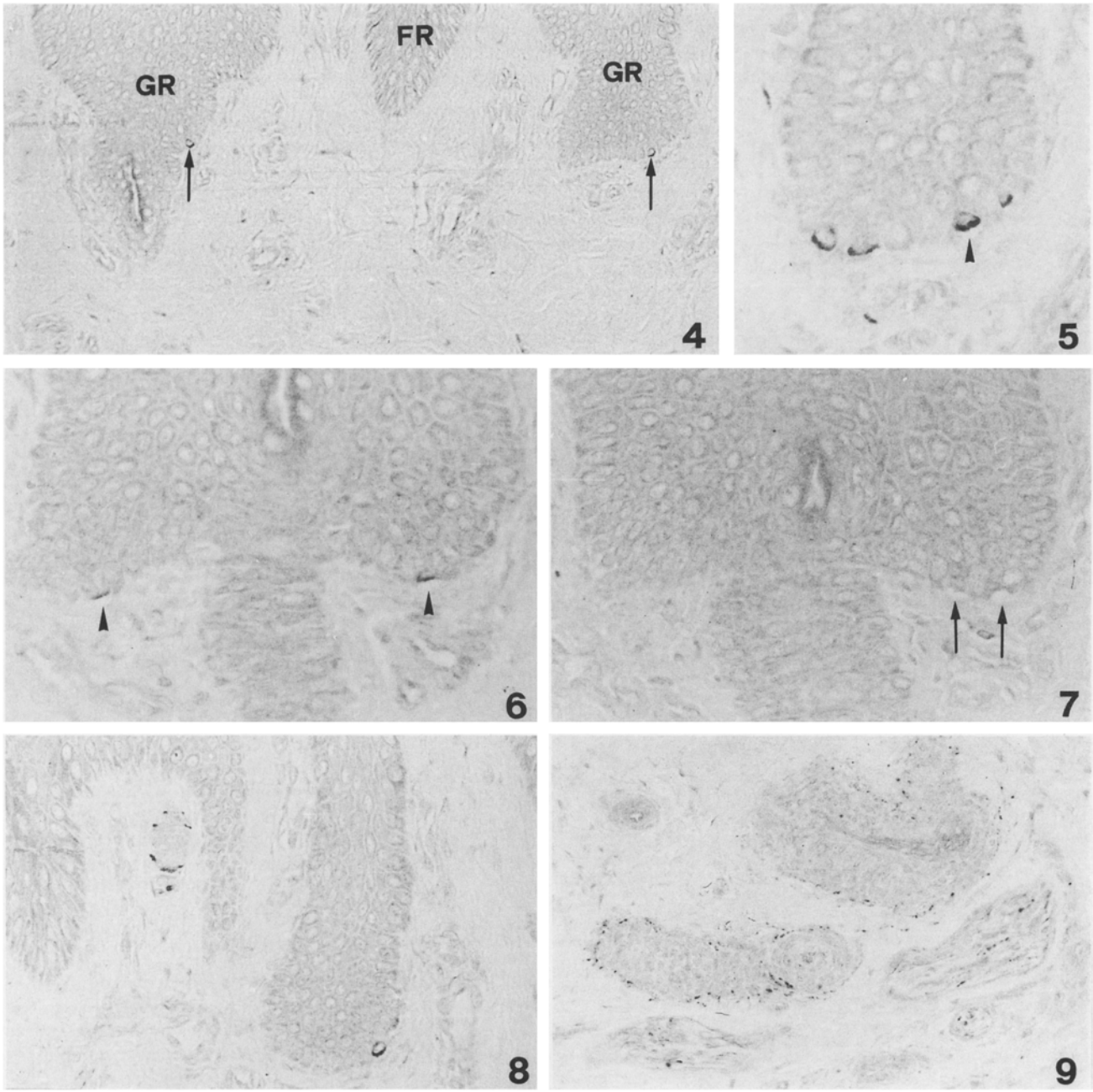
Fig. 2a–b. Pig snout, sinus hair follicles (vibrissae). **a** In vibrissae, CGRP-immunoreactive Merkel cells are located in the external root sheath (170 ×). **b** Higher magnification shows that in this case immunostaining is most intense on the epithelial side of Merkel cells close to the nerve terminals (*arrowheads*) (350 ×)

Fig. 3a–c. Pig snout skin, CGPR-immunoreactive nerve fibres. **a** An immunoreactive nerve fibre with varicosities in the epidermis (275 ×). **b** Nerve bundles containing immunostained nerve fibres located at dermal level (150 ×). **c** Nerve fibres containing CGRP forming a network around a glomus (250 ×)

Results

Pig snout

In pig snout epidermis, all Merkel cells were immunoreactive for CGRP, being localized at the tips of rete ridges, chiefly grouped in large clusters (Fig. 1a). Immunostaining was most intense on the basal side of the Merkel cells, which was in contact with associated nerve endings (Fig. 1b). The nerve endings were not immunoreactive for CGRP and appeared as clear discs situated below the Merkel cells (Fig. 1b).



Figs. 4–9. Human fingertip

Fig. 4. In the human fingertip, CGRP-immunoreactive Merkel cells (*arrows*) are generally isolated at the tip of glandular ridges (*GR*) (*FR* = Fixation ridges) (210 ×)

Fig. 5. Four CGRP-immunoreactive Merkel cells situated in the basal layer of an epidermal ridge. Immunoreactivity is stronger on the dermal side of the cells facing the unimmunostained nerve ending (*arrowhead*). Several dermal nerve fibres are also immunostained (530 ×)

Fig. 6. Immunostained Merkel cells symmetrically located on both sides of an eccrine sweat gland duct. Note that the terminal discs (*arrowheads*) are not CGRP-positive (500 ×)

Fig. 7. Non immunostained Merkel cells (*arrows*) in a semiserial section incubated with preabsorbed antiserum (500 ×)

Fig. 8. Merkel cell and nerve fibres of a Meissner's corpuscle with immunostaining for CGRP (300 ×)

Fig. 9. Nerve bundles with some CGRP containing nerve fibres, which also appear surrounding blood vessels (175 ×)

Sinus hair follicles (vibrissae) also exhibited CGRP-containing Merkel cells, which were arranged in a row in the external root sheath (Fig. 2a). In this case the intensity of immunostaining was greatest in the part of the Merkel cells facing into the follicle, which was now the part closest to the nerve terminals (Fig. 2b) (unlike the epidermis, vibrissae have Merkel cells interposed between the basal membrane and the nerve ending).

Nerve fibres exhibiting immunoreactivity for CGRP were found *1)* in the superficial dermis below ridges or run-

ning into papillae (Fig. 1b); *2)* penetrating the epidermis and ending as free terminals (Fig. 3a); *3)* forming part of nerve bundles at dermal or hypodermal level (Fig. 3b); and *4)* surrounding blood vessels (mainly arteriovenous anastomoses of glomus type) (Fig. 3c).

Human fingertip

Merkel cells immunostained for CGRP were found isolated or constituting small groups at the base of glandular epidermic ridges, but not at fixation ridges (Fig. 4). In humans, as in the epidermal Merkel cells of pig snouts, immunostaining was most intense in the basal part of the epidermal Merkel cells, close to the nerve endings (Fig. 5). Merkel cells were sometimes located symmetrically about the ducts of ecrine sweat glands (Fig. 6). Like pig specimens, all human Merkel cells were immunostained for CGRP. Antiserum specificity controls did not exhibit immunostaining (Fig. 7).

Intraepidermal nerve terminals associated with Merkel cells did not exhibit immunoreactivity (Figs. 5 and 6), but some nerve terminals containing CGRP were found in the superficial dermis contiguous to ridges (Fig. 5), in the Meissner's corpuscles (Fig. 8) or, more deeply, in nerve bundles and around vascular structures (Fig. 9).

Discussion

Our results are the first immunohistochemical evidence of CGRP immunoreactivity in the Merkel cells of pig snout and human fingertip skin. Morphological criteria for optical identification of Merkel cells are largely described (Merkel 1875; Munger 1965; Munger et al. 1971; Smith 1977; García-Caballero et al. 1989) and allow us to state that all Merkel cells were immunostained for CGRP. This fact is in agreement with the finding of Alvarez et al. (1988b) that CGRP immunoreactivity is present in all Merkel cells of hard palate, upper lip hairy skin touch domes and vibrissae of cats. Immunostaining of the Merkel cells of both species occurs on the side of the cell adjacent to the neurite; there is no immunoreaction in the nerve disc of the Merkel cell-neurite complex. CGRP has also been observed in the Merkel cell-neurite complexes of cat skin, where it is likewise located in the Merkel cells rather than in their associated nerve terminals (Alvarez et al. 1988b).

If the finding (Lauweryns and Van Ranst 1987) that CGRP is contained in the dense-cored granules of pulmonary epithelial neuroendocrine cells closely related to Merkel cells also holds good for Merkel cells, then the intracellular location of immunostaining in this study would be explained by the greater numbers of dense-cored granules in the stained areas.

Although CGRP appears to be expressed predominantly in neural tissue (Kawai et al. 1985), there are also known to be CGRP-positive neuroendocrine dispersed cells in various species (Cadieux et al. 1986; Uddmann et al. 1986; Kuramoto et al. 1987; Lauweryns and Van Ranst 1987; Scheuermann et al. 1987; Alvarez et al. 1988a). Our results contribute to knowledge of the neuroendocrine dispersed system (Håkanson and Sundler 1983) in the species studied. It has been generally accepted that this cell-neurite complex has a mechanoreceptive function, but the molecular biology of the Merkel cell is still unclear, and a number of roles are currently entertained (Hartschuh and Weihe 1980; Gottschaldt and Vahle-Hinz 1981, 1982; Hartschuh et al. 1984; Tachibana et al. 1984; Diamond et al. 1986). The presence of CGRP in Merkel cells supports the hypothesis that they act via paracrine pathways.

A number of CGRP immunoreactive intraepidermal nerve fibres ending as free terminals were found in pig snout skin. The nerve terminals forming part of Merkel cell-neurite complexes, on the other hand, were CGRP-negative. Similar CGRP-immunoreactive intraepidermal free nerve endings have also been found in cat skin (Alvarez et al. 1988a). Previous studies on mammalian skin innervation have demonstrated the presence of substance P in some nerve fibres in human skin (Dalsgaard et al. 1983) and obtained positive reactions for bombesin, somatostatin, substance P and vasoactive intestinal polypeptide in feline and porcine skin (O'Shaughnessy et al. 1983).

In human fingertips we found CGRP-immunoreactive nerve fibres forming part of Meissner corpuscles. If it is legitimate to extrapolate from Halata and Munger's description (1983) of the nerve fibres surrounding Meissner's corpuscles in the monkey, the peptidergic nature of these fibres and their peripheral location in the corpuscles suggest they probably correspond to nerve free endings. Alvarez et al. (1988a) obtained similar morphological results using antisera to CGRP and substance P in the cat skin.

Perivascular CGRP fibres were frequently seen around vascular beds; they were most numerous around arteries and veins. CGRP fibres were even more in evidence around arteriovenous anastomoses in the upper parts of the reticular dermis, intimately associated with tightly packed smooth muscle fibres. A similar perivascular distribution of CGRP nerve fibres has been shown to exist and to regulate blood flow in the respiratory, genitourinary and gastrointestinal tracts of the guinea pig (Uddmann et al. 1986).

In conclusion, the CGRP immunoreactivity of the Merkel cells of the skin in the species studied suggest that CGRP may act via paracrine pathways, although further pharmacological and physiological investigations will be required to determine precisely what function(s) are subserved by this peptide. The role of CGRP-immunoreactive nerve fibres ending as free terminals is probably to transduce sensorial stimuli. Since vasodilator effects of synthetic CGRP have been observed in human skin after intradermal injection in volunteers (Brain et al. 1985), our immunohistochemical observations of CGRP-positive perivascular innervation suggest that CGRP fibres may regulate local blood flow in the skin of the species studied.

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