SENSORY NEUROMEDIATORS IN HUMAN SKIN: ROLE IN INFLAMMATION AND OTHER DISORDERS

by

Joanna Wallengren

Department of Dermatology
Lund University
General Hospital
Malmö, Sweden

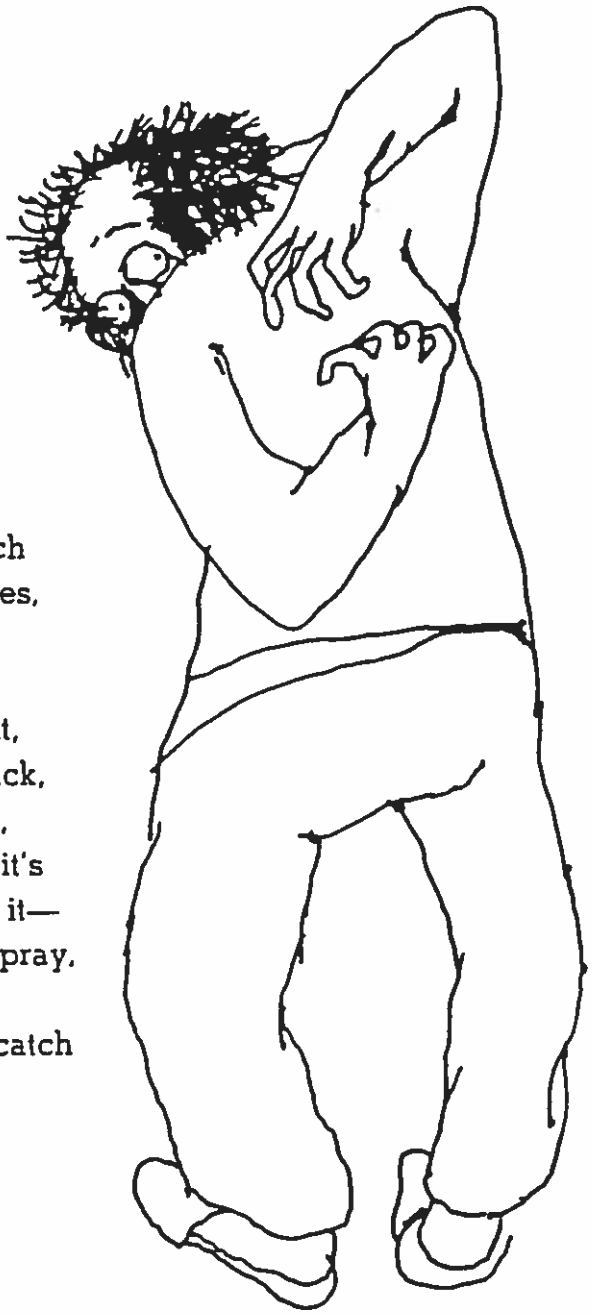
Malmö 1990

To my family

Nils-Olof and Sara

and to my parents

Dana and Henry



UNSCRATCHABLE ITCH

There is a spot that you can't scratch
Right between your shoulder blades,
Like an egg that just won't hatch
Here you set and there it stays.
Turn and squirm and try to reach it,
Twist your neck and bend your back,
Hear your elbows creak and crack,
Stretch your fingers, now you bet it's
Going to reach—no that won't get it—
Hold your breath and stretch and pray,
Only just an inch away,
Worse than a sunbeam you can't catch
Is that one spot that
You can't scratch.

Shel Silverstein (reprinted with kind permission of Cape publ.).

This thesis is based on the following papers, which will be referred to in the text by the Roman numerals given below.

I. Occurrence and distribution of neuropeptides in human skin. An immunocytochemical and immunochemical study on normal skin and blister fluid from inflamed skin. Wallengren J, Ekman R, Sundler F. Acta Derm Venereol (Stockh) 1987: 67: 185-192.

II. Physiological factors influence substance P evoked flare in human skin. Wallengren J, Håkanson R, Andrén-Sandberg Å. Submitted.

III. Effects of substance P, neurokinin A and calcitonin gene-related peptide in human skin and their involvement in sensory nerve-mediated responses. Wallengren J, Håkanson R. European Journal of Pharmacology 1987: 143: 267-273.

IV. Effects of capsaicin, bradykinin and prostaglandins in human skin. Wallengren J, Håkanson R. Submitted.

V. Substance P and vasoactive intestinal peptide in bullous and inflammatory skin disease. Wallengren J, Ekman R. Möller H. Acta Derm Venereol (Stockh) 1986: 66: 23-28.

VI. Treatment of notalgia paresthetica with topical capsaicin. Wallengren J, J Am Ac Derm. In press.

VII. Some neuropeptides as modulators of experimental contact allergy. Wallengren J, Möller H. Contact Dermatitis 1988: 13: 351-354.

VIII. Substance P antagonist inhibits immediate and delayed type hypersensitivity reactions. Wallengren J. Brit J Derm. Accepted for publication.

CONTENTS

Abbreviations and definitions

CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
DNCB	dinitrochlorobenzene
DYN	dynorphin
GAL	galanin
ic	intracutaneous
ICC	immunocytochemistry
-IR	immunoreactive/-ity
MED	minimal erythema dose - the lowest dose of UVB resulting in a homogenous, well marginated erythema after 24 hours
NKA	neurokinin A
NPY	neuropeptide Y
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
PHI	peptide histidine isoleucine
PNS	peripheral nervous system
PP	pancreatic polypeptide
PUVA	psoralen plus UVA (long-wave ultraviolet radiation (320-300 nm)
PYY	peptide YY
RIA	radioimmunoaassay
SK	Substance K, a former name for NKA
SP	substance P
UVB	medium-wave ultraviolet radiation (279-320 nm)
VIP	vasoactive intestinal polypeptide

Aims of the thesis

1. To describe the occurrence and distribution of different neuropeptides in normal human skin.

2. To measure the concentrations of neuropeptides in blister fluid and the concentrations of such peptides in the plasma of patients with inflammatory skin disorders.

3. To investigate the mode of action of tachykinins, CGRP, bradykinin and capsaicin in human skin.

4. To evaluate the significance of various physiological factors on neurogenic inflammation in response to a standard challenge.

5. To elucidate how histamine and prostaglandin E_2 participate in neurogenic inflammation.

In the course of the investigation new research data prompted studies on:

6. The possible role of SP in immediate and delayed hypersensitivity reactions using a tachykinin antagonist (Spantide) as a pharmacological tool.

7. The possible role of sensory C-fibres in notalgia paresthetica using capsaicin as a pharmacological tool.

Introduction

NEUROGENIC INFLAMMATION

The term neurogenic inflammation implies that the the nervous system contributes to the symptoms of inflammation. The first report to support this concept was published by Goltz (1874) who observed that stimulation of the sciatic nerve produced vasodilatation in the innervation territory of this nerve. Later Stricker (1876) made similar observations following stimulation of the dorsal roots. In 1901 Bayliss showed that antidromic stimulation of a primary afferent nerve evoked vasodilatation in the skin. The term *"axon reflex"* was introduced to describe the fact that the nerve impulse travels not only centrally (orthodromic impulse traffic) but also peripherally (anti-dromic impulse traffic). Bruce (1913) demonstrated that some of the symptoms of acute inflammation caused by the application of mustard oil to the skin could be blocked by local anesthesia and concluded that the symptoms developed only in the presence of an intact sensory nerve supply. Lewis (1927) studied the response evoked by local injury to the skin and characterized it as a *triple response* with local redness, distant flare and wheal. The distant flare and spreading hyperalgesia around the site of injury was shown to be due to local nervous mechanisms and to depend on primary afferent neurons. The skin response to injury resembles the reaction to intradermal injection of *histamine*, which is thought to be a mediator in the inflammatory process. Vasodilatation and increased vascular permeability is produced by stimulation of chemosensitive small diameter afferent fibres (C-fibres) (Hinsey & Gasser 1930), which are associated with both nociception and axon reflexes (Celander & Folkow 1953). Dale (1935) suggested that antidromic vasodilatation reflects the peripheral release of the same neuromessenger substance that mediates central transmission in the C-fibres. Many of the criteria for being a mediator in sensory nerves are fulfilled by substance P (SP) (Lembeck & Holzer 1979). C-fibres store several other neuropeptides and some of them like neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) coexist with SP in the same nerves (Sundler et al

1985). Jancsó and collaborators suggested in 1967 that pain-provoking chemical irritants such as capsaicin (the pungent agent of hot pepper) produce inflammation through stimulation of the C-fibres and can serve as a model in the study of neurogenic inflammation. The mechanism behind the generation of the distant flare - the main feature of neurogenic inflammation is still a matter of discussion (Holzer 1988) . A functional coupling between nerve fibres and mast cells has been proposed by Lembeck and Gamse (1982). There is an intimate anatomical relationship between mast cells and sensory nerve fibers (Skofitsch et al 1985).

ENDOGENOUS INFLAMMATORY MEDIATORS

Histamine

Lewis (1927) demonstrated that the triple response to injury in the skin is mimicked by intradermal injection of histamine. Furthermore the flare component of the triple response (arteriolar vasodilatation) was found to depend on an intact nervous network within the skin. Histamine was found to be stored in mast cells by Riley and West in 1953. Kiernan (1975) showed that nerve stimulation, that causes vasodilatation, degranulates mast cells in the skin. Histamine receptor antagonists (H_1- histamine receptors) block neurogenic vasodilatation (Hägermark et al 1978).

Bradykinin

Fray and Kraut (1928) discovered a hypotensive substance in the pancreas which they named kallikrein (Greek for pancreas). Werle (1937) found that kallikrein was an enzyme that catalyzed the formation of an active serum substance called kallidine. In 1949 Rocha e Silva et al found that incubating blood with trypsin generated an agent that contracted the guinea pig ileum. The contraction was slower than that induced by histamine, hence the name "bradykinin". In 1956 Rocha e Silva identified bradykinin as a peptide and together with Andrade he purified it by chromatography. The peptide was sequenced and synthesized

(Boissonas et al 1960). In 1970 Roche e Silva suggested that kinins should be considered "as a class of tissue hormones locally released from diffuse stores in the body not having specialized glands of secretion and being rapidly inactivated at the site of release". The result of the balance between simultaneous production and inactivation is that kinins exist in circulating blood in very low concentrations (the half-life of kinins in blood is very short). Prekallikreins and kallikreins have been found in exocrine glands such as the pancreas, kidney, intestine, salivary and sweat glands (for review see Regoli and Barabé 1980) and are released by the stimulation of the autonomic nervous system, by drugs and other stimuli. The effects of bradykinin seem to include excitation of peripheral sensory nerves. Thus, Juhlin and Michaëlson (1969) found that flare induced by bradykinin in the skin is inhibited by local anesthetics. Bradykinin has been found to cause partial depletion of neuropeptides in C-fibres (Håkanson et al 1987).

Prostaglandins

In 1930 Kurzrok and Lieb observed that the human uterus responded with either strong contraction or strong relaxation upon instillation of fresh human semen. Three years later Goldblatt in England and von Euler in Sweden studied the strong smooth-muscle stimulating activity of human seminal plasma. Von Euler prepared lipid extracts from vesicular glands of male sheep and found the activity to be associated with the fraction containing lipid soluble acids. The active factor was called prostaglandin (for a review see Bergström et al 1968). The isolation in pure crystalline form of the first two prostaglandins, now called PGE_1, and $PGF_{1\alpha}$, was reported 1960 by Bergström et al. All prostaglandins contain 20 carbon atoms and have the same carbon skeleton, "prostanoic acid". Prostaglandins are products of cyclo-oxygenase activity on arachidonic acid and they are produced by many mammalian tissues. $PGE_{2\alpha}$ is produced mainly by macrophages and neutrophils and PGD_2 mainly by mast cells and basophils. Prostaglandins E_1 and E_2 are effectively removed from the blood during one passage through the vascular system (Ferreira and Vane 1967). The actions of different prostaglandins often differ greatly. PGE_2 relaxes smooth muscle, is a

bronchodilatator and enhancer of cutaneous permeability. It induces vasodilatation in the skin and enhances histamine-, serotonin- and bradykinin-evoked oedema, pruritus and pain (Williams 1983).

NEUROPEPTIDES

Substance P (SP)

In 1931 von Euler and Gaddum reported that extracts of equine brain and intestine contained a hypotensive and spasmogenic factor which seemed to be distinct from all biologically active principles known at that time. The active ingredient was prepared in the form of dry powder, displaying chemical and biological characteristics which distinguished it from histamine. Early findings suggested that the new compound, provisionally named substance P (SP, P for powder) (Gaddum & Schild 1934), might be a protein or a peptide (von Euler 1936). The occurrence and tissue distribution of SP could be studied by the preparation of crude tissue extract followed by bioassay. Besides the gastrointestinal tract in the rabbit (von Euler 1936) and gold fish (Gaddum & Szerb 1961) SP was demonstrated in different parts of the central nervous system (more in the grey than in the white matter and more in the subcortical areas than in the cortex) (Pernow 1983) and also in peripheral nerves. After section of the sciatic nerve, the concentration of SP was reduced greatly in the distal stump, while it increased in the proximal part (Holton 1958). SP was isolated from bovine hypothalamic tissue and chemically characterized (Chang 1971) and synthetized (Tregear et al 1971). The synthesis of SP made it possible to develop highly specific immunohistochemical and radioimmunological techniques for detailed studies of its distribution and release.

The distribution of SP has been established using the indirect immunofluorescence method of Coons et al (1958). The role of SP as a putative neurotransmitter was put forward after the finding of large amounts of SP in the dorsal

horn of the spinal cord. Dorsal root sectioning in the cat led to depletion of SP from the dorsal horn of the spinal cord. Instead SP accumulated on the ganglionic side of the lesion (Takahashi and Otsuka 1975). SP is present in primary sensory nerves with their cell bodies in the spinal and trigeminal ganglia (Hökfelt et al 1975). In the skin SP-containing fibres are thin and unmyelinated (C-fibres) and supply free nerve endings just beneath the epithelium. SP nerve fibres have been found to form a network around sweat glands and blood vessels (Hökfelt et al 1975). In human digital skin SP-immunoreactive fibres were found both in free nerve endings and in Meissner' s corpuscles (Dalsgaard et al 1983). SP occurs in most tissues in the body, e.g. the urogenital tract, gastrointestinal tract and respiratory tract (Sundler et al 1985). SP is thought to be involved as a mediator in sensory processes of various types, including nociception (Henry 1977) and inflammation (Hägermark et al 1978).

SP analogues and antagonists

SP and structurally related peptides such as neurokinin A (NKA) comprise a group of peptides naturally occurring in mammals termed tachykinins. Other tachykinins, among them eledoisin, kassinin and physalaemin, have been isolated from submammalian species (octopod salivary glands or amphibian skin) (Ersparmer 1981). All tachykinins share a common C-terminus.

A series of SP analogues has been studied with respect to their ability to block SP-mediated responses. Several such analogues have been shown to block the effects of SP both in vitro (rabbit iris sphincter) (Bynke et al 1984) and in vivo (e.g. SP-induced ocular effects in the rabbit) (Holmdahl et al 1981, Beding-Barnekow 1990).

Experiments on human skin have provided information on the effects of antagonists on both flare and wheal of SP (Piotrowski et al 1984). Like SP itself the antagonists also release histamine from mast cells. Spantide, (D-Arg^{1},D-Trp$^{7.9}$, Leu^{11}) SP, combines a high antagonistic potency with a weak spasmogenic

effect and poor histamine releasing properties (Folkers et al 1984 a).

Neurokinin A (NKA)

NKA was first isolated from the porcine spinal cord (Kimura et al 1983,Kangawa et al 1983, Minamino et al 1984). Amino acid analysis and sequencing revealed NKA to be a decapeptide, belonging to the tachykinin family. Nawa et al (1983) determined through gene cloning the primary structure of two bovine SP precursors. In one of them the sequence of NKA was found, in addition to the sequence of SP; in the other SP only was found. Dalsgaard et al (1985) found SP and NKA to coexist in primary sensory nerves in the rat. The biological activity of NKA was investigated using the gut-contracting assay as a tool (Folkers et al 1984b) and found to be 81% of the activity of SP. NKA is also active with regard to gall-bladder contraction, protein extravasation, hypotension and bronchial smooth muscle spasm (for a review see Tatemoto et al 1985).

Calcitonin gene-related peptide (CGRP)

CGRP is a 37 amino acid peptide. It is encoded by the same gene that codes for calcitonin. Alternative processing of the RNA transcripts from the calcitonin gene results in the production of two distinct mRNAs encoding either calcitonin or CGRP (Amara et al 1982). CGRP has been found in sensory neurons in the trigeminal ganglion and in the spinal ganglia. Conceivably, CGRP plays an important role in the sensory nervous system. It coexists with SP and NKA in sensory nerve cell bodies (Sundler et al 1985). It is co-localized with SP also in the nerve fibers of the skin of guinea pigs (Gibbins et al 1985). In human skin CGRP induces slowly developing local reddening (duration of several hours) (Brain et al 1985) and not a triple response as do the coexisting peptides SP and NKA. There are reports suggesting an enhancing effect of CGRP on the response to other mediators of increased vascular permeability (Brain & Williams 1985, Gamse & Saria 1985).

Vasoactive intestinal peptide (VIP)

VIP was isolated from porcine intestine by Said and Mutt 1970 and identified as an octacosapeptide (Mutt and Said 1974). Using immunohistochemical techniques VIP was found in nerve fibres and neuronal cell bodies in the gastrointestinal tract (Bryant et al 1976, Larsson et al 1976) and in the hypothalamus (Larsson et al 1976). VIP is also present in peripheral nerve structures including parasympathetic ganglia, the vagus nerve, pancreatic ganglia, urogenital tract, the cerebral vessels (for a review see Said 1988) and in nasal mucosa and the tracheobronchial tree (Uddman et al 1978).

VIP received its name because of its hypotensive actions and its potent vasodilatory activity on the splanchnic, coronary and pulmonary vascular beds. VIP relaxes the smooth muscle of blood vessels (causing vasodilatation). VIP and acetylcholine coexist in postganglionic parasympathetic nerve fibres supplying e.g. salivary glands (Lundberg et al 1981, Sundler et al 1986).

In the skin VIP-immunoreactive nerve fibres have been found to supply the arteries and arterial portions of arteriovenous anastomoses (Hartschuh et al 1984). In eccrine sweat glands of the human axilla and in Meibomian glands VIP-containing nerve fibres seemed to innervate glandular cells (Hartschuh 1983). VIP has been suggested to play a role in thermoregulation through vasodilatation and regulation of sweat gland function.

Somatostatin

Somatostatin is a tetradecapeptide that was discovered in 1973 (Brazeau et al 1973) as a hypothalamic factor with an inhibitory effect on the release of growth hormone. It was subsequently demonstrated in other tissues such as the gastrointestinal tract. It is a highly potent inhibitor of peptide hormone release and cell proliferation (see Johansson 1988). Somatostatin is localized to spinal ganglia (about 10% of the cell bodies are somatostatin-immunoreactive) and spinal cord (dorsal horn). Somatostatin occurs in nerve fibres in the skin (Johansson 1988).

Neuropeptide Y (NPY)

In 1982 Tatemoto et al reported that porcine brain and gut contained large amounts of peptides resembling pancreatic polypeptide (PP) and found that the brain peptide differed from that in the gut. The PP-like peptide in the brain was named neuropeptide Y (NPY) and that in the gut PYY.

NPY consists of 36 aminoacids and has been found in neuronal elements not only in the brain but also in the periphery. In addition, it occurs in cells of the adrenal medulla. A dense supply of NPY-immunoreactive fibres was found in the respiratory tract, the heart, gut, pancreas and the urogenital tract. Blood vessels within these and other structures received a rich supply of NPY fibres (Sundler et al 1986). Since many nerve cell bodies in the sympathetic ganglia were found to store NPY, it was suggested that noradrenergic sympathetic neurons contained NPY (Lundberg et al 1982) which was confirmed by sequential immunostaining for NPY and dopamine-B-hydroxylase (DBH), a marker for noradrenergic neurons (Lundberg et al 1982).

NPY suppresses the stimulated release of noradrenaline via a presynaptic action (Lundberg et al 1982). The vasomotor response to exogenously applied noradrenaline is greatly potentiated by NPY (Edvinsson et al 1984, Ekblad et al 1984, and Wahlestedt et al 1985).

Coexistence of neuropeptides.

Sensory neurons are rich in neuropeptides. Among peptides known to exist in such neurons are SP, NKA, CGRP, VIP, galanin (GAL), dynorphin (DYN), Somatostatin and cholecystokinin (CCK). There is increasing evidence that some of these neuropeptides coexist in the same neurons. Clearly the chemical coding of the individual neurons vary greatly. The different populations of sensory neurons with differing chemical coding (coexisting neuropeptides) might have different targets. Populations of SP, CGRP, DYN and CCK - containing nerve fibers have been shown in the skin of guinea pig (Gibbins et al 1987) and VIP/PHI - and CGRP/SP - containing nerve fibers in sweat glands in the cat

(Lindh 1987). Not surprisingly SP and NKA coexist in primary sensory neurons (Dalsgaard et al 1985).

NOXIOUS AGENTS

Capsaicin

Capsaicin, the active principle of hot pepper of the genus capsicum exhibits broad bioactivity (for a review see Fitzgerald 1983, Buck and Burks 1986). It affects neuronal structures and causes bronchospasm and gastrointestinal and dermatologic irritation.

Capsaicin seems to produce irrversible degeneration of C-fibres in rats only if administred up to 10 - 14 days of age (Jancsó, Kiraly 1980). A depletion of 50-60% of SP in the dorsal horn of the spinal cord has been reported by most authors following neonatal capsaicin treatment 50 mg/kg (for a review see Fitzgerald 1983). The depletion of primary afferent neurons persists to at least 7 months (Cuello et al 1981). The best effects are seen when capsaicin is administred on day 2 or day 5 after birth and is less pronounced if the treatment is on day 10 (Gamse et al 1980). When administered to adult rats capsaicin depletes SP from small sensory neurons and their terminals in the CNS and PNS but the nerves do not degenerate.

Capsaicin applied locally to the sciatic nerve blocks axonal transport of peptides in small fibres. Accumulation of SP and somatostatin proximal to the site of application occurs after 24 h (Gamse et al 1982). The effect is specific for sensory C fibres and is not due to a local damage of those fibres since they still conduct action potentials (review Fitzgerald 1982) and the fibres are not degenerated (Ainsworth et al 1981). Capsaicin also depletes CGRP, NKA and VIP from sensory nerve endings (for review see Holzer 1988). Topical capsaicin pretreatment inhibits axon reflex vasodilatation caused by thermal injury (Carpenter and Lynn 1981) and elevates heat and pain thresholds (Anand et al 1983).

The SP antagonist, Spantide, has been described to inhibit capsaicin induced effects (Håkanson et al 1987, Maggi et al 1989). The same effect may be achived by ruthenium red which binds irreversibly to calcium channels where capsaicin is thought to act via a specific receptor (Chahl 1989).

NEUROPEPTIDES AND SKIN DISEASE

There is increasing evidence that the nervous system influences skin diseases. The data supporting this theory are summarized in Table I.

NOTALGIA PARESTHETICA

Notalgia paresthetica ("noton" from Greek, meaning "back") was first described by Astwazaturrow in 1934. The term refers to a neuropathy involving the dorsal primary divisions of the spinal nerves. Pruritus, often coming in attacks, occurs on a well defined, sometimes pigmented patch just medial to the scapular border at the level T2 through T6. (Fig. 1). Necrotic keratinocytes in the epidermis have been claimed to be a characteristic feature of notalgia paresthetica (Weber et al 1988) (Fig. 2). In one study electromyographic examination of patients with notalgia paresthetica revealed evidence of paraspinal nerve damage localized to the T2 through T6 level (Maysey 1981). There are other reports of normal histological and electromyographical findings in patients with notalgia paresthetica (Marcusson et al 1990). Topical corticosteroids and antihistamines are ineffective. PUVA has also been proved to be ineffective. (E Tegner personal communication). Transcutaneous nerve stimulation does not relieve the itch (Fjellner and Hägermark 1978).

The aim of the present study was to explore the possibility that neuropeptides may be involved in the pathophysiology of notalgia paresthetica. Paroxysmal itch may result from a sudden local release of neuropeptides. Depletion of neuro-

Table I. Neuropeptides and skin disease

Author	*Disease*	*Evidence*
Bernstein et al (1986)	*Psoriasis*	*Improves following treatment with capsaicin*
Naukkarinen et al (1987)	*Psoriasis*	*Increased density of SP-containing nerve fibres in lesions*
Pincelli et al (1990)	*Psoriasis*	*Increased density of VIP fibres and content of VIP in lesions*
Fantini et al (1990)	*Atopic eczema*	*Increased VIP content in lesions*
Pincelli et al (1990)	*Atopic eczema*	*Increased density of SP-containing and NPY-containing nerve fibres in lesions*
Bernstein et al (1987)	*Postherpetic neuralgia*	*Improves following treatment with capsaicin*
Bucci et al (1988)	- " -	- " -
Bernstein et al (1989)	- " -	- " -
Tóth-Kása et al (1983)	*Cold and heat urticaria*	*No urtication following capsaicin desensitization*
Jancsó et al (1985)	*Cold and heat urticaria*	*No urtication following capsaicin desensitization*
- " -	*Herpes Zoster*	*No flare response to histamine or mustard oil*
Wallengren et al (1987)	*Cold urticaria Dermographism*	*Increased SP- and VIP- levels in blister fluid*
Marks (1989)	*Apocrine chromhidrosis*	*Successfull treatment with capsaicin*

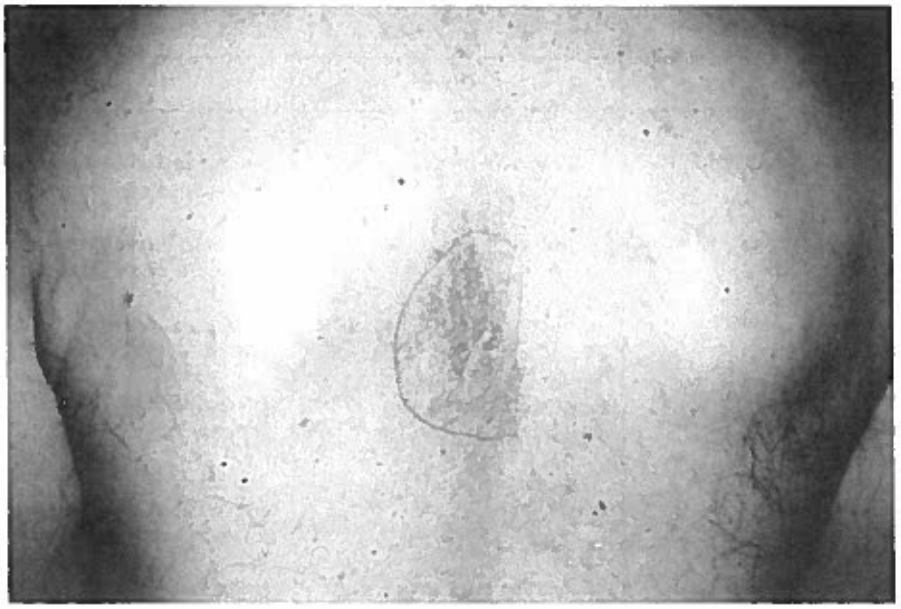


Fig. 1. *Pigmented patch of notalgia paresthetica on the back.*

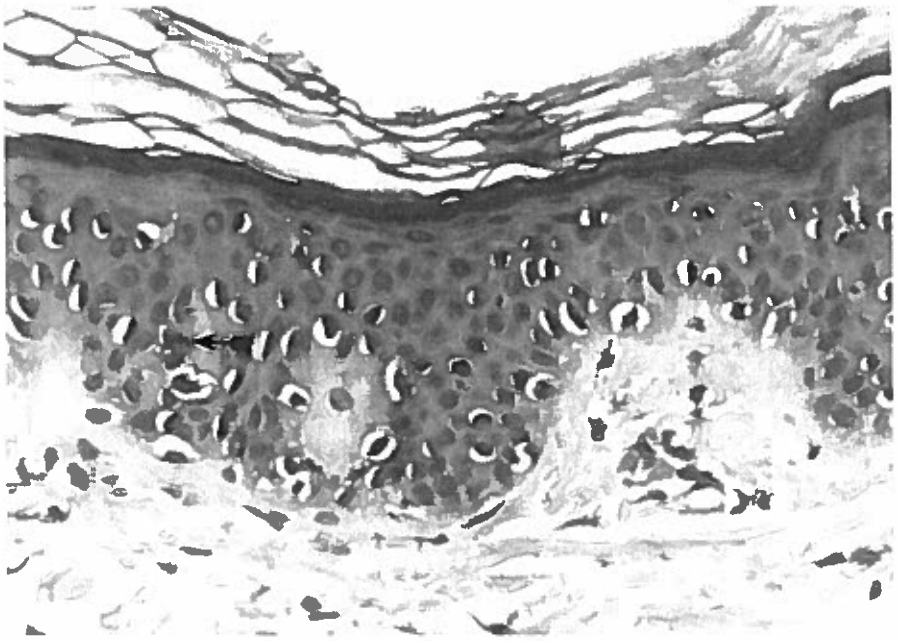


Fig. 2. *The characteristic feature of notalgia paresthetia, a necrotic keratinocyte, labelled with an arrow (photo was kindly provided by ass prof C Lindström).*

peptides from peripheral nerves might thus relieve itching. Neuropeptides can be released from sensory fibers by capsaicin. When applied to the skin capsaicin releases SP and CGRP; when applied repeatedly it depletes the sensory nerves of their content of SP and CGRP and impairs their reaccumulation (Buck and Burks 1986). Topical capsaicin 0.025% (Zostrix®) has been successful in the treatment of postherpetic neuralgia (Bernstein et al 1987, 1989) and it was decided to test its effect on notalgia paresthetica.

INTERACTION BETWEEN THE NERVOUS SYSTEM AND THE IMMUNE SYSTEM

Different neuropeptides have been suggested to regulate immunologic responses. In order to prove the concept of a "neuro-immune axis" one must demonstrate the release of the messenger peptide in the proximity of an immune effector cell.

This is fulfilled by only few neuropeptides (for reviews see O' Dorisio 1988, Bienenstock et al 1988, Payan, Goetzl 1988). Nerve endings occur in the vicinity of various components of the immune system (Blalock 1984).

High affinity receptors for SP have been identified on human lymphocytes (Payan et al 1984). SP stimulates proliferation of T-lymphocytes (both helper and suppressor cells) (Payan et al 1983, Stanisz et al 1986) a finding which, however, has been questioned by Reusch et al 1988. SP stimulates also immunoglobulin synthesis (Stanisz et al 1986, Payan et al 1984, Scicchitano et al 1988). Stimulation of mast cells and basophils by SP leading to histamine release is also a part of the immunologic process.

High affinity VIP binding sites on human mononuclear cells have been demonstrated by several investigators (for a review see O' Dorisio 1988). VIP inhibits T-lymphocyte proliferation (O' Dorisio et al 1985, Scicchitano et al 1987), NK cell activity (Rola-Pleszczynski et al 1985) and inhibits synthesis of

immunoglobulins IgA, IgM and IgG (Stanisz et al 1986).

High affinity receptors for somatostatin have been demonstrated on human lymphocytes (O' Dorisio 1988). Somatostatin inhibits proliferation of T-lymphocytes (for a review see Payan et al 1984) and immunoglobulin synthesis (Stanisz et al 1986).

Material and Methods

BRIEF DESCRIPTION OF THE STUDIES

I. The concentration and distribution of SP, somatostatin, VIP, CGRP, and NPY in human skin were investigated by immunocytochemistry and radioimmunoassay (RIA). The samples were obtained from normal skin and from different regions. RIA was performed on pooled tissue samples from several regions (fingers, toes, axillas and thighs) and on tissue fluid from spontaneous blisters on inflamed skin.

II. The influence of various physiologic factors on neurogenic inflammation was studied using the dermal response to locally injected SP as a model. The different physiologic parameters were as follows: different skin regions of the body, day and night, environmental temperature, age, sex and stress.

III. The mode of action of the C-fibre neuropeptides SP, NKA and CGRP was studied in skin pretreated with the histamine liberator compound 48/80, the H_1 - antagonist mepyramine, or the local anesthetic lidocaine.

IV. Actions and interactions of several putative mediators of inflammation such as SP, histamine, bradykinin and prostaglandin (PGE_2) were studied. The mode of action of the neuropeptide releasing agent capsaicin was explored. Pretreatment with lidocaine, compound 48/80, mepyramine, indomethacin, atropine or ketanserin (a serotonin antagonist) was performed.

V. The concentrations of SP and VIP in the plasma of patients with disseminated skin diseases and pruritus were measured by radioimmunoassay (RIA). The same analyses were performed on the fluid from spontaneous

blisters and from suction blisters induced on inflamed as well as normal skin.

VI. Notalgia paresthetica is a neuralgia manifested as attacks of itch and pain in a distinct skin area close to the medial border of the scapula (T2 - T6). The possibility that neuropeptides may be involved in the pathophysiology of this disorder was explored by treating the patients with capsaicin for about 4 - 6 weeks. Subsequently they were under observation for about a year.

VII. The role of neuropeptides in allergic contact dermatitis was investigated. SP, VIP, somatostatin, an SP antagonist (Spantide) or saline were injected 10 min after the injection of nickel sulphate and in some cases 24 h and 48 h after nickel injections. All reactions were evaluated 72 h after the first injection.

VIII. The role of SP in immunologic and non-immunologic, immediate and delayed inflammatory reactions was investigated using the SP antagonist, Spantide. Spantide was found to inhibit the SP-evoked response in the skin. Spantide was then injected ic on the volar aspect of the forearm prior to the following challenges: epicutaneous application of benzalkonium chloride (irritant delayed reaction), tuberculin (immunologic delayed reaction), benzoic acid (non-immunologic contact urticaria, NICU), different foods and latex (in patients with immunologic contact urticaria) as well as UVB irradiation.

SUBJECTS

The characteristics of the individuals included in the present studies are shown in table II.

Table II. Some characteristics of the individuals studied

Paper	Cathegory	Study	Age range	Mean age	Number
I.	healthy subj	biopsies, ICC	24 - 89	62	24
	autopsy	biopsies, ICC, RIA	69 - 86	74	6
	patients with skin disease	blister fluid, RIA	17 - 97	60	22
II.	healthy subj	pharmacological study	5 - 60	26	33
	athletes	- " -	18 - 24	22	10
III.	healthy subj	- " -	20 - 35	26	51
IV.	healthy subj	- " -	24 - 60	30	37
V.	patients, generalized skin disease	Plasma, RIA	17 - 91	57	56
	skin disease with spontaneous blisters	Blister fluid, RIA	13 - 90	57	39
	suction blisters on non-diseased skin	Blister fluid, RIA	25 - 93	69	14
	suction blisters on diseased skin	Blister fluid, RIA	20 - 79	55	12
VI.	patients (notalgia paresthetica	treatment with capsaicin	37 - 73	58	10
VII.	patients (nickel allergy)	treatment with Spantide	27 - 67	39	12
VIII.	healthy subj induction of different inflammations	treatment with Spantide	17 - 50	34	16
	patients (contact urticaria)	- " -	19 - 46	27	7

STUDIES ON OCCURENCE AND DISTRIBUTION OF NEUROPEPTIDES IN HEALTHY AND DISEASED HUMAN SKIN (I, V)

Biopsies

Punch biopsies with a diameter of 3 mm were taken from different skin regions of healthy volunteers under local anesthesia (prilocain and adrenalin). The biopsies from autopsy material consisted of 4 - 6 mm punches.

Blood samples

Blood samples was drawn from the antecubital vein of patients with various disseminated inflammatory skin diseases. Blood samples were collected in tubes containing sodium EDTA 1 mg/ml and Trasylol (500 KIU/ml), immediately cooled with ice and centrifuged. The plasma was then stored at - 20^{O} C until extraction for determination of SP and VIP.

Blister fluid

Blister fluid from spontaneous blisters due to different skin diseases (I, V) and from suction blisters (see below) (V) was collected with a syringe and placed in ice. The fluid was stored at - 20^{O} C until analysis by RIA.

Suction blisters

Suction blisters provide a model for the study of skin blistering (Kiistala 1976). They resemble morphologically those occurring in pemphigoid (at the level of epidermal - dermal junction). Suction blisters have also been used to collect tissue fluid. The protein content of suction blister fluid is 20 - 40 % of that of the serum.

Suction blisters were induced on the forearm skin (ventral area) by a vacuum pressure of 4.5 mWS. In each patient eight 4 mm blisters were provoked within 60 - 90 min. Blister fluid was collected immediately.

Immunocytochemistry

The biopsies were fixed by immersion overnight in 4% buffered formaldehyde, pH 7.2. They were then rinsed repeatedly in sucrose - enriched (10%) buffer and frozen on dry ice. Cryostat sections (10 μm thickness) were processed for immunocyto-chemistry using antibodies against the following neuropeptides: SP, CGRP, VIP, NPY and somatostatin. The antigen-antibody reaction was visualized by fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (TRITC)-conjugated antirabbit IgG and examined in a fluorescence microscope equipped with two different filtersettings for viewing FITC and TRIC, respectively. For the monoclonal SP antibody FITC-labelled antimouse IgG was used as second antibody. The simultaneous demonstration of SP- and CGRP- immunoreactive nerve fibers in the same tissue section was performed using the monoclonal SP antibody and FITC- labelled second antibody followed by application of the CGRP antiserum and TRITC-labelled second antibody.

Extraction of SP, VIP, CGRP, NPY and somatostatin from skin tissue

Tissue samples (punch biopsies) from normal skin of out-patients at the Dermatology Clinic and from autopsy subjects were frozen and stored at -20^{0} C. After boiling in 0.5 M acetic acid for 15 min the specimens were homogenized (Polytron 1-2 min). After centrifugation at 2.000 x g for 15 min. the super-natants were lyophilized. For RIA, the freeze-dried material was dissolved in buffer and centrifuged before assay.

Immunochemical and chromatographic methods

RIA was performed on extracts of skin and on unextracted blister fluid from spontaneous and suction blisters (see below) and on plasma from patients with disseminated skin diseases. All samples were assayed in serial dilutions.

Immunoreactive SP was determined using rabbit antiserum SP-2 (I) or SP-8 (V) in a final dilution of 1:125,000. 125 I-Tyr^{8}-SP was used as tracer. The smallest

amount detectable is 3 pmol/l (4 pg/ml) after concentration of the samples. The rabbit antiserum does not cross-react with bombesin, gastrinreleasing peptide, eledoisin and neurokinin A. The intra- and interassay variations are 4 and 8.3 % (n = 20), respectively.

For the RIA of VIP we used a rabbit antiserum (No 7852, Milab, Malmö, Sweden) in a final dilution 1:40,000. The detection limit is 4 pml/l (13 pg/ml) and the intra- and interassay variations are 4.0 and 8.5 % (n = 20), respectively. The antiserum recognizes the N-terminal 15 amino acid sequence of VIP. The antiserum does not cross-react with cholecystokinin, secretin, GIP, PHI or glucagon.

Immunoreactive CGRP was determined using a rabbit antiserum (R 8429, Milab, Malmö, Sweden) (raised aginst synthetic rat CGRP) in a final dilution of 1:75,000 and with 125 I (Tyr^0)-CGRP as a tracer. Human CGRP cross-reacts fully in this RIA. The detection limit is 20 pmol/l, the intra-assay variation is < 6 % and the interassay variation < 15% in the range 20 - 200 pmol/l.

NPY was measured by using an antiserum against natural porcine NPY conjugated to bovine serum albumin. This antiserum cross-reacts fully with porcine PYY but not at all with PP, GIP, PHI, VIP and secretin. The detection limit is 12 pmol/l, intra-assay variation is < 6% and inter-assay variation < 10%.

Immunorective somatostatin was determined using rabbit antiserum (No K18, Milab, Malmö, Sweden) in a final dilution 1:25.000. It does not cross-react with any other known neuropeptide besides cyclic somatostatin (100%) Tyr^1 (Somatostatin) (100%) expressed as Somatostatin 15-28 equivalents. The detection limit is 5 pmol/l. Intra-assay variation was 6 % and inter-assay variation 12 %. All samples were assayed in serial dilutions (duplicate samples).

In order to partly characterize the SP- and VIP-immunoreactive material, high performance liquid chromatography (HPLC) was used. The elution of SP and VIP and the columns used are described in detail in paper V.

PHARMACOLOGICAL STUDIES (II, III, IV, VI, VII, VIII)

All experiments were performed by the same person. Except in paper IV and VI all drugs were administered by intracutaneous injections (ic) usually in a volume of 0.05 ml. Capsaicin was injected ic in most experiments (IV) and applied topically in others in order to to induce desensitization by repeated doses.

DRUGS USED IN THE EXPERIMENTS

Substance P, CGRP, NKA, Somatostatin, VIP - Peninsula Laboratories, California, USA
Bradykinin - Peninsula Europa, St Helen, Merseyside, UK
Spantide - Ferring AB, Sweden

Atropine
Capsaicin
Compound 48/80 (histamine liberator)
Histamine dihydrochloride
Mepyramine (H_1 - receptor antagonist)
PGE_2

} Sigma Chemical Co (St Louis, USA).

Lidocain - Xylocain® - Astra Pharmaceuticals, Södertälje, Sweden
Ketanserin (serotin antagonist) - Janssen Pharmaceuticals, Belgium
Indometacin - Confortid®, Dumex, Sweden

All substances were dissolved in physiological saline. In order to dissolve capsaicin a few drops of Tween 80 and ethanol was added. Topical administration of capsaicin: - 0.1% solution in ethanol (IV), - Zostrix® cream (0.025% capsaicin), Genderm, (Northbrook, IL) (VI).

CHEMICALLY INDUCED INFLAMMATIONS (VII, VIII)

Immunologic delayed reactions.

Patients with contact dermatitis to nickel (verified by patch test) participated in the investigation (VII). Nickel sulphate was injected ic once at 5 sites on the volar aspects of the forearms. The dose chosen for patients with strong test reactions was 30 nmol and for those with moderate reactions 50 nmol. The nickel injections were followed by injections of solutions in physiological saline of synthetic SP, VIP, somatostatin or Spantide. All reactions were evaluated 72 h after the first injection.

Tuberculin reaction. 0.1 ml of 2 TU PPD (Statens Serum Institut, Copenhagen, Denmark) was injected ic and the response area was examined after 72 h.

Irritant delayed reaction

Benzalkonium chloride 1% in water solution was applied to the skin of the ventral part of the forearm by using Finn Chamber® on Scanpor®. The compound was applied for 48 h and the reaction examined after another 24 h.

Ultraviolet evoked reaction (UVB)

The irradiation source was a 150W xenon arc (Osram XBO). The exposure time was 16-25 sec. The erythema was evaluated after 24 h.

Immunologic contact urticaria

Patients with contact urticaria to food, fruit and latex were challenged by scratching the volar aspect of the arm superficially with blood lancet and applying the substances by van der Bend square chambers® for 20 min. Evaluation was made 5 - 15 min after removal of the chambers.

Non - immunologic contact urticaria (NICU)

5% benzoic acid dissolved in petrolatum was applied to the skin by Finn Chambers® on Scanpor® for 45 min. The urticarial reaction was registered immediately after removal of the chambers (for a review see the thesis by Lahti, 1980).

TREATMENT OF NOTALGIA PARESTHETICA (VI)

Treatment with Capsaicin 0.025% (Zostrix® cream) was used to release neuropeptides from sensory nerve fibres and to prevent their reacummulation. Zostrix® was applied five times a day for one week and three times daily for about 3 - 4 weeks.

EVALUATION

The flare and wheal responses to neuropeptides, histamine, bradykinin, PGE_2 (II, III, VIII) and the response to challenge with nickel sulphate (VII) were evaluated planimetrically. The flare reactions were outlined and traced on transparent plastic film when maximal, i.e. 5 min after the injection (if not otherwise stated). The wheal response was assessed in the same way when maximal i.e. 15 min after the injection (if not otherwise stated). The outlined areas were cut out and measured,

Two different score systems were used for evaluation of the inflammatory reactions. The reactions to UVB and benzalkonium chloride were evaluated as follows:

1. erythema.
2. erythema and infiltration.
3. erythema and strong infiltration.
4. erythema, strong infiltration and vesicles.

The infiltrated response to tuberculin, benzoic acid and immunologic contact urticaria were scored as follows with regard to the mean of two angle diameters:

1. < 1 cm
2. < 1.5 cm
3. < 2.5 cm
4. ≥ 2.5 cm

The evaluation of the treatment of notalgia paresthetica with Zostrix® was made once weekly and based on the frequency and intensity of the itch. After the treatment the patients were under observations for a year.

STATISTICAL METHODS (II, III, IV, VI, VII)

In most of the experiments, comparison of mean areas for flare and wheal between controls and individuals subjected to different pretreatments/conditions were carried out by Wilcoxon' s signed rank test and Wikcoxon' s rank sum test. Students t-test was used when suitable. Friedman' s test was used in paper VI. The significance levels were as follows * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.