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CHARACTERIZATION AND BIOCHEMICAL MECHANISMS OF THE NEUROTOXIC ACTIONS OF CAPSAICIN

The University of Arizona PH.D. 1982

CHARACTERIZATION AND BIOCHEMICAL MECHANISMS OF THE NEUROTOXIC ACTIONS OF CAPSAICIN

by

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A Dissertation Submitted to the Faculty of the

COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Matthew Steven Miller

entitled Characterization and biochemical mechanisms of the neurotoxic actions of capsaicin.

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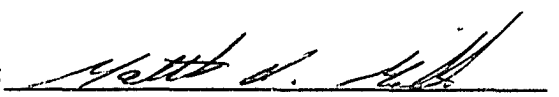
SIGNED: 

To my wife Mary Jo and daughter Jessica

ACKNOWLEDGMENTS

I would like to express my sincere graditude to Drs. Thomas F. Burks and I. Glenn Sipes for giving me the freedom to learn and the insight to succeed.

I would also like to thank Dr. Klaus Brendel for his assistance in preparing dihydrocapsaicin, Dr. Ryan Huxtable for his help in measuring spinal cord amino acid levels, and Drs. David Kreulen and Henry Yamamura for their consistent support throughout this endeavor.

Special thanks are extended to my fellow students James McDougal and James Galligan who were always willing to offer constructive criticism and Stephen Buck who made measurement of substance P levels possible.

TABLE OF CONTENTS

TABLE OF CONTENTS--continued

TABLE OF CONTENTS--continued

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--continued

LIST OF ILLUSTRATIONS--continued

LIST OF ILLUSTRATIONS--continued

LIST OF TABLES

ABSTRACT

Capsaicin, the primary pungent component of hot peppers, produced chemogenic and thermal antinociception within two hours after administration to adult guinea pigs (2-8 mg/kg). Antinociception lasted in excess of 10 days. In addition, in somewhat higher doses (4-25 mg/kg s.c.) capsaicin also depleted the putative peptide neurotransmitter, substance P, from primary afferent neurons. Depletion of substance P by capsaicin did not occur until at least one day after capsaicin treatment and the onset of antinociception. Antinociception produced by capsaicin appeared to be a result of bioactivation and covalent binding of capsaicin to the distal ends of sensory neurons. Capsaicin depleted substance P from sensory nerves by inhibiting the rate of substance P synthesis by 48 percent. Inhibition of substance P synthesis by capsaicin occurred with some degree of specificity as the rate at which total protein was synthesized was unchanged. The biochemical mechanism by which capsaicin altered substance P synthesis involved alterations in the retrograde axoplasmic transport of nerve growth factor. Doses of capsaicin which depleted substance P also inhibited the retrograde axoplasmic transport of nerve growth factor. Inhibition of the retrograde transport of nerve growth factor by capsaicin preceded substance P depletion. Supplementation of guinea pigs with mouse nerve growth factor completely prevented

capsaicin-induced substance P depletion. It is concluded that capsaicin depletes substance P from primary afferent neurons of the adult guinea pig by altering the availability of NGF. The data support a role for NGF in the normal maintenance of neuropeptide levels in some sensory neurons in the adult animal.

INTRODUCTION

Many plants in the genus Capsicum contain within their fruits an intensely sharp and pungent compound known as capsaicin (Fig. 1). Species of red pepper which contain capsaicin in concentrations ranging from 0.45-1.82 mg/g dry weight are a common ingredient of human diets throughout the world (Glinsukon et al., 1980). For example, the daily intake of capsaicin by the average adult in Thailand has been reported to be approximately 50 mg/day, while in India daily intake of capsaicin is limited to approximately 7.5 mg/day (Srinivasan et al., 1980).

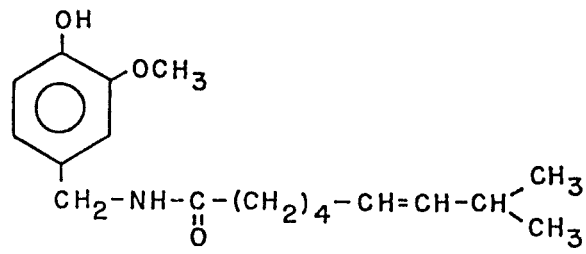


Figure 1. Structure of capsaicin

Gastrointestinal Effects of Capsaicin

Upon ingestion, capsaicin produces an intense burning sensation within the oral cavity. A similiar response is produced following either cutaneous or subcutaneous application of capsaicin (Lille and

Ramirez, 1935). Following ingestion, capsaicin is completely absorbed from the gastrointestinal tract. Experiments in rabbits have shown that no capsaicin is excreted in the feces following an oral dose of 5 mg/kg (Lee, 1963a,b). Additionally, 60-80 percent of a capsaicin dose (0.6-1.0 mg) instilled into the ligated small intestine of rats disappears within 1 hour (Srinivasan et al., 1980).

Once in the gastrointestinal tract, capsaicin produces several pharmacologic effects. Limlomwongse, Chartauchawong and Tonayai (1979) measured gastric acid secretion and blood flow in gastric mucosa of anesthetized rats which were treated with capsaicin. Capsaicin (0.05-0.1 mg/kg p.o.) increased both gastric acid secretion and mucosal blood flow in a dose-dependent manner. No changes in systemic blood pressure were observed. Capsaicin-induced gastric acid secretion was completely abolished by atropine (0.02 mg/kg) or hexamethonium (20 mg/kg). Hence, it appears that capsaicin alters gastric acid secretion and mucosal blood flow by stimulating cholinergic parasympathetic pathways.

Nopanitaya and Nye (1974) investigated the effects of capsaicin on duodenal absorptive cells in rats. Intragastric or intraduodenal capsaicin administration (1.0 mg/kg) produced a direct toxic effect on duodenal absorptive cells. Light and electron microscopic studies revealed that histological changes in duodenal absorptive cells occurred within two minutes of capsaicin administration. The changes became progressively worse, reaching a maximum effect in animals which were exposed to capsaicin for 45-60 minutes. Tissue damage was

characterized by swollen mitochondria with disorganized cristae. Increased numbers of free ribosomes and dilatation of endoplasmic reticulum and Golgi complexes were evident. Nuclear material was clumped and marginated at the nuclear envelope. The histological changes produced by capsaicin were not unlike those produced by neomycin, methotrexate, aminopterin, puromycin and cycloheximide. Since these compounds have diverse mechanisms of action it was concluded that capsaicin produced its toxicity on intestinal absorptive cells by a non-specific mechanism.

The actions of capsaicin on intestinal nutrient absorption has been extensively investigated. Capsaicin stimulates intestinal glucose absorption at low intralumenal concentrations (0.07 mg/ml). At higher concentrations (0.14-0.21 mg/ml) capsaicin inhibits intestinal absorption of glucose (Monsereenusorn and Glinsukimon, 1978). Capsaicin-induced inhibition of glucose absorption was associated with a decrease in intracellular adenosine triphosphate (ATP) in the intestinal mucosa. Addition of ATP to the mucosal side of capsaicin-treated intestinal preparations restored gluose absorption rates to control values. This suggested that capsaicin inhibited intestinal glucose absorption by reducing the ATP content of intestinal mucosal cells, possibly by altering mitochondrial oxidative phosphorylation. Further evidence that capsaicin altered mitochondrial function by inhibition of oxidative phosphorylation was provided by Monsereenusorn (1979). It was demonstrated that capsaicin (0.14 mg/ml) added to everted rat intestine inhibited glucose transport while utilization of glucose and lactic acid production were increased.

Systemic Toxicity of Capsaicin

Glinsukon et al. (1980) investigated the acute toxicity of capsaicin in five species. LD_{50} values demonstrated that the guinea pig was most susceptible to capsaicin (1.1 mg/kg i.p.), while rats and mice were slightly less sensitive (6.5-13.2 mg/kg i.p.) and rabbits and hamsters were considerably less susceptible to capsaicin (50 mg/kg i.p.). Rats which were administered lethal doses of capsaicin demonstrated hyperactivity and convulsions within 1-2 minutes of capsaicin injection. Siezures induced by capsaicin were similiar to grand mal siezures produced by pentamethylenetetrazol and were completely blocked by pentobarbital anesthesia. The apparent cause of death in every case was repiratory failure. At necropsy, visceral organs appeared hyperemic and increased peritoneal fluid was observed in animals treated with intraperitoneal capsaicin.

The systemic toxicity produced by repeated capsaicin administration was investigated by Lee (1963a,b). Adult rabbits were fed red pepper at a dose of 5 g/kg/day (approximately 5 mg/kg/day of capsaicin) for a period of 1 year. At termination, it was found that the weight of the heart, liver, lung, spleen, kidneys and adrenal glands were increased when compared to control animals. Histological analysis revealed degenerative and necrotic changes in these same tissues.

Effects of Capsaicin on Cardiovascular and Respiratory Systems

Capsaicin has well characterized actions on the cardiovascular and respiratory systems. Lille and Ramirez (1935) demonstrated that capsaicin produced hypotension and variable respiratory effects in anesthetized dogs. Subsequent studies by Porszasz, Gyorgy and Porszasz-Gibiszer (1955) revealed that intravenous capsaicin produced hypotension, bradycardia and transient apnea in both cats and dogs. The apnea was consistently followed by an increased respiratory rate which is thought to be result of direct stimulation of central nervous system respiratory centers by capsaicin (Porszasz, Such and Porszasz-Gibiszer, 1957). In the cat, it is not uncommon for capsaicin to initially produce hypotension, which is then followed by hypertension. The pressor effect of capsaicin in cats appears to be of peripheral origin since decapitation, ganglionic blockade and sympathetic blockade did not prevent the hypertension (Porszasz et al., 1955). In 1955, Toh, Lee and Kiang reported that either vagotomy or destruction of the sinus nerve prevented capsaicin-induced hypotension and apnea in dogs. This led Toh et al. (1955) to postulate that capsaicin stimulated carotid sinus baroreceptors and pulmonary stretch receptors through a vagal reflex, resulting in hypotension and apnea. This postulate was further supported by Porszasz et al. (1957) who reported that blockade of carotid sinus chemoreceptors with acetic acid failed to block capsaicin-induced hypotension. However, application of the local anesthetic cocaine to the carotid sinus did block the hypotension. Subsequent electrophysiologic studies (Coleridge,

Coleridge and Kidd, 1964) conclusively demonstrated the capsaicin excited vagal baroreceptor afferents. Porszasz et al. (1957) and Coleridge et al. (1964) found that capsaicin was most effective in producing hypotension, apnea and bradycardia when injected directly into the pulmonary artery. This suggested that capsaicin produced its hypotensive effects by acting at a site within the pulmonary vascular bed as well as at the arterial baroreceptors.

Thermoregulatory Action of Capsaicin

It has been known for many years that capsaicin is capable of producing profound changes in thermoregulation. When administered parenterally, capsaicin produces a rapid dose-dependent (0.2-10 mg/kg s.c.) fall in body temperature (Szolcsanyi and Jancso-Gabor, 1973; Cabanac, Cormareche-Leydier and Poirier, 1976). This hypothermia is accompanied by vasodilitation, salivation and a decrease in oxygen consumption. In fact, capsaicin administration mimics the effects of directly heating the hypothalamus (Hammel, 1968). Pretreatment of rats with a single dose of capsaicin (75 mg/kg s.c.) prior to a second dose of capsaicin (10 mg/kg s.c.) completely abolishes the thermoregulatory response to the second dose (Szolcsanyi and Jancso-Gabor, 1973). Additionally, prior capsaicin treatment, or desensitization, markedly inhibited the decreases in oxygen consumption produced by a single dose of capsaicin. The mechanism by which capsaicin produced hypothermia was investigated by Jancso-Gabor, Szolcsanyi and Jancso (1970). It was postulated that capsaicin first stimulated, then desensitized

hypothalamic warmth detectors. Jancso-Gabor et al. (1970) supported this postulate with the following data:

1. Direct injection of 0.5-25 ug of capsaicin into the preoptic area of the hypothalamus produced a rapid dose-dependent fall in body temperature (0.2-2.0 ^{o}C). Upon repeated hypothalamic injection of capsaicin the hypothermic response gradually disappeared.
2. Rats which were desensitized with hypothalamic injections of capsaicin lost the ability to regulate body temperature when placed in a warm environment (37-39 ^{o}C).
3. Direct heating of the anterior hypothalamus by diathermy produced a greatly attenuated thermoregulatory response in rats which were pretreated with capsaicin.

The mechanism by which capsaicin impairs hypothalamic warmth detectors is not known. Jancso and Wolleman (1977) demonstrated that adenylate cyclase activity was increased in the preoptic area of the hypothalamus following systemic treatment with capsaicin. This enhanced adenylate cyclase activity was inhibited by in vitro addition of capsaicin or serotonin. It was concluded that capsaicin altered thermal regulation through a mechanism which involved aberrations in adenylate cyclase activity. In summary, it appears that capsaicin alters thermoregulation by inducing a biochemical or structural hypothalamic lesion which in turn renders hyothalamic heat detectors non-functional.

Effects of Capsaicin on Nociception

As previously mentioned, capsaicin exerts a violent excitatory action on sensory nerve endings following oral, cutaneous or subcutaneous administration (Jancso, Jancso-Gabor and Takats, 1961). After this initial excitation, capsaicin produces a desensitization which extends to all chemical irritants. Repeated cutaneous capsaicin treatment produces an area, corresponding to the site of administration, which is completely insensitive to chemical irritants (Jancso, 1966). There is, however, no apparent reduction in sensitivity to physical stimuli such as pressure. This phenomenon is easily produced in human subjects (Jancso, 1960).

The desensitization to chemogenic pain produced by capsaicin results from a lesion to specific pain receptors. Porszasz and Jancso (1957) using electrophysiologic techniques demonstrated that rat cutaneous nerve endings previously desensitized with capsaicin (4, 8, 15, 15 mg s.c. over 3 days) did not initiate action potentials in response to chemical stimulation with capsaicin (10^{-6} M), nicotine (10^{-5} M) or acetylcholine (10^{-5} M). Sensory neurons from non-desensitized animals responded readily to the chemical stimuli.

A similiar experiment has been conducted on human facial skin (Jancso, 1966). One cheek was repeatedly painted (10 times) with a 0.5 percent solution of capsaicin in ethanol. Initial applications produced intense burning pain, local hyperemia, and edema. With repeated application, both the intensity and duration of the symptoms decreased until finally no response was detected. If both cheeks were

painted with 10 percent ammonia, only the cheek not pretreated with capsaicin responded with pain and hyperemia. The capsaicin-treated cheek was asymptomatic. Reapplication of ammonia 24 hours later produced results identical to those of the previous day. Hence, capsaicin appears to be capable of selectively, and possibly irreversibly, lesioning cutaneous chemogenic pain receptors in rats and humans.

Parenteral administration of capsaicin to rats or guinea pigs results in a generalized desensitization of peripheral sensory neurons to chemogenic nociceptive stimuli (Jancso, 1966). This desensitization of chemogenic neurons lasts for months in the rat and years in the guniea pig (Jancso, 1960).

Measurements of thermal nociception in capsaicin-treated rats has yielded conflicting results. Hayes and Tyers (1980) reported that capsaicin pretreatment in adult rats produced elevated chemical and pressure threshold but no change in thermal nociception when measured 5 days after the last dose of capsaicin. Rats which were treated with capsaicin as neonates, however, demonstrated significantly elevated thresholds for thermal nociception (Nagy et al., 1980; Holtzer et al., 1979). Further characterization of this phenomenon revealed that capsaicin pretreatment prior to the tenth day of life resulted in alterations in thermal nociception, while treatment after day 10 produced no changes in thermal sensitivity (Holtzer et al., 1979). The age-related alterations in capsaicin-induced thermal analgesia have been related to neuronal degeneration in the dorsal spinal cord which

appears to be correspondingly age-related (Holtzer et al., 1979). The mechanism of this phenomenon is unknown.

Since capsaicin alters sensory transmission, Joo, Szolcsanyi and Jancso-Gabor (1969) studied the cellular architecture of dorsal roots and dorsal root ganglia of rats which were pretreated with capsaicin on two consecutive days (15 and 20 mg/kg s.c.). The small dark staining B cells in dorsal root ganglia demonstrated ultrastructural changes which were characterized by swollen mitochondria in which organized cristae were absent. These ultrastructural changes were still apparent 2 months after capsaicin treatment. The data prompted further investigations concerning the effects of capsaicin on the central processes of primary sensory afferent neurons.

In 1975, Jancso and Knyihar investigated the effect that systemic desensitization with capsaicin (50, 100 mg/kg/day s.c.) had on fluoride resistant acid phosphatase (FRAP) staining in the spinal cord. FRAP staining has been suggested to be functionally related to the processing of nociceptive information within the spinal cord (Knyihar, 1971; Knyihar, Laszlo and Tornyos, 1974). FRAP activity was found to be decreased as early as 2 days after the first injection of capsaicin. Five days after the first capsaicin injection FRAP activity disappeared completely and did not reappear until almost 7 months later. It was concluded that capsaicin had an action on the terminal ends of the central processes of primary sensory neurons.

Sensory Neurotransmitters

Currently there are two principal candidates for the role of neurotransmitter in sensory neurons. These are the amino acid L-glutamate (Fig. 2) and the peptide substance P.

L-Glutamate

L-glutamate is present in both dorsal root ganglia and dorsal spinal cord in high concentrations (Duggan and Johnson, 1970; Johnson and Aprison, 1970) and exerts a powerful depolarizing action on secondary sensory neurons in both the spinal cord and brain stem (Johnson, 1972; Curtis and Johnson, 1974). Recent studies have,

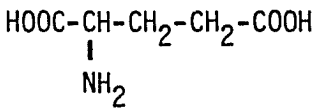


Figure 2. Structure of the putative sensory neurotransmitter L-glutamate.

however, raised doubts that L-glutamate serves as a sensory neurotransmitter. Schon and Kelley (1974) demonstrated that ^{3}H-L-glutamate was actively transported into glial cells surrounding sensory nerve terminals but not into the sensory neurons themselves. The absence of a reuptake system to replenish neuronally released L-glutamate suggested that L-glutamate was not being released by sensory neurons. Schon and Kelley (1974), however, suggested that

neuronally released L-glutamate was rapidly sequestered in glial cells and then slowly transferred to adjacent sensory neurons. A similiar mechanism is thought to exist in GABAergic cerebellar purkinje cells. Therefore, the glial cell-neuron symbiosis postulated by Schon and Kelley (1974) is not without precedent. However, the evidence supporting a role for L-glutamate as a neurotransmitter in sensory neurons is, at best, weak. The role for substance P, as a sensory neurotransmitter, though not compelling, is substantially stronger than that for L-glutamate.

Substance P

Substance P was first discovered in 1931 by von Euler and Gaddum who were at the time investigating an unidentified potent hypotensive agent in extracts of equine intestine and brain. Gaddum and Schild (1934) named the agent substance P because most activity was found in the powder which resulted when acetone extracts of equine tissue were dried. Von Euler (1936) then demonstrated that substance P was peptide in nature. For many years it was thought that substance P actually consisted of a series of biologically active peptides. Early studies regarding the distribution of bioassayable substance P revealed that substance P existed in sensory neurons in relatively large quantities (Lembeck, 1953). This led Lembeck (1953) to speculate that substance P may be acting as a neurotransmitter in sensory neurons. Investigating the physiologic role of substance P in sensory neurons was not possibly, however, due to the lack of pure substance P or specific antibodies to substance P.

In 1970 Chang and Leeman isolated substance P from bovine hypothalamus and characterized its primary amino acid structure. It was determined that substance P consisted of a single peptide containing 11 amino acids (Fig. 3).

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Figure 3. Primary structure of substance P.

Subsequently, substance P isolated from cat dorsal roots and equine intestine was characterized and was found to be structurally identical to that isolated from bovine hypothalamus (Chang, Leeman and Niall, 1971; Studer, Trzeciak and Lergier, 1973; Takahashi and Otsuka, 1975). Hence, the structure of substance P appears not to be species dependent. Characterizing the primary structure of substance P allowed for synthetic preparation of substance P and for the development of specific antibodies to substance P.

The availability of specific antibodies to substance P allowed Hokfelt, Ljungdahl et al. (1977) to demonstrate the exisitence of substance P containing nerve terminals in dorsal horn of spinal cord and peripheral nerve endings (skin and tooth pulp). In addition to occurring in sensory neurons, substance P has also been demonstrated in neurons of the hypothalamus, caudate nucleus, stria terminalis,

amygdala, habenula nucleus, raphe nuclei and the periaqueductal central gray (Hokfelt, Johansson et al., 1977; Ljundahl, Hokfelt and Nilsson, 1978, Cuello, Emson et al., 1978; Cuello, Del Fiacco and Paxinos, 1978).

The evidence supporting a role for substance P in the transmission of nociceptive information is extensive. Early experiments prior to the isolation of substance P by Chang and Leeman (1970) showed that bioassayable substance P was synthesized in dorsal root ganglia and axonally transported to the dorsal horn of the spinal cord (Holton, 1958). Substance P disappeared from the dorsal horn of the spinal cord after sectioning the dorsal roots (Otsuka, Konishi and Takahashi, 1972). Futhermore, substance P was found to be concentrated in synaptosome fractions of brain (Ryall, 1964). These data are consistent with a localization of substance P in nerve terminals. Otsuka et al. (1972) showed that substance P which had been isolated from the dorsal roots of bovine spinal nerves depolarized motor neurons in the isolated rat spinal cord. Addition of substance P to isolated spinal cord resulted in deploarization of ventral roots. Substance P was found to be 1000-9000 time more potent than L-glutamate. Furthermore, the excitatory effects of substance P on spinal motor neurons persisted even when synaptic transmission in the spinal cord was blocked by low calcium or tetrodotoxin. This indicated that substance P was acting directly on motor neurons and was not exciting dorsal horn interneurons (Konishi and Otsuka, 1974). Intracellular recordings from spinal cord motor neurons during application of substance P demonstrated that substance P-induced depolarization as a

result of increases in membrane resistance. It was postulated that substance P increased membrane resistance by reducing membrane K^+ conductance (Phillis, 1978).

Henry (1976) showed that the spinal neurons which are excited by iontophoretic application of substance P are the same neurons which respond to noxious stimuli. Since substance P is found in small diameter C or A delta sensory nerve fibers (Hokfelt, Johansson et al., 1977; Ljungdahl et al., 1978; Cuello, Del Fiacco et al., 1978), it seems likely that substance P may play a physiologic role in the function of nociceptive sensory neurons. Strong evidence supporting this postulate comes from the observations of Olgart et al. (1977) who observed that removal of the tooth pulp in cats resulted in a loss of substance P containing nerve endings in the trigeminal nucleus. These substance P containing processes represent the central processes of substance P containing trigeminal neurons which degenerate after tooth extraction (Gobel and Binck, 1977). Since tooth pulp reportedly contains only pain sensitive fibers, the findings of Olgart et al. (1977) further suggested that substance P was associated physiologically with primary afferent neurons which respond to nociceptive stimuli.

The existance of opiate receptors on primary afferent nerve terminals was demonstrated by La Motte, Pert and Snyder (1976). La Motte et al. (1976) concluded that opiate receptors were on the central endings of primary afferent neurons because the number of opiate receptor binding sites in the dorsal horn decreased after sectioning of the dorsal roots. Experiments carried out on the rat spinal trigeminal

nucleus in vitro (Jessell and Iversen, 1977) suggest that some of these presynaptic receptors may be located on substance P containing terminals because opiate receptor agonists, such as morphine, enkephalins and beta-endorphin, abolish the K^+ evoked release of substance P from superfused slices of trigeminal nucleus. It was suggested that enkephalin containing neurons are located adjacent to substance P containing primary afferent nerve endings in the spinal cord. Opiates would then modulate, by presynaptic inhibition, the transmission of nociceptive information at the level of the spinal cord. The apparent association between substance P and enkephalin containing fibers is not confined to the spinal cord but extends to other CNS area which are believed to be involved in additional aspects of the response to nociceptive stimuli. These include the raphe nuclei, ventral tegmental area, septum and amygdala (Hokfelt, Ljungdahl et al., 1977). It remains to be seen if substance P release in these areas can also be suppressed by opiate receptor agonists.

Apart from its effects on spinal cord neurons, substance P has also been tested on neurons in the cerebral cortex, substantia nigra and amygdala (Ben-Ari, Le Gal La Sal and Levesque, 1978; Davies and Dray, 1976; Phillis and Limacher, 1974; Walker et al., 1976). In the majority of cases, substance P excited neurons, although some depression was observed in the substantia nigra and cerebral cortex (Phillis, 1978). Substance P was found to be very potent when tested on cerebral cortex neurons (Phillis and Limacher, 1974), where it may act presynaptically to evoke acetylcholine release (Phillis, 1978). It

is not yet clear if the slow onset of substance P excitation, which is normally observed in such studies, is due to inadequate iontophoretic techniques for substance P (ie. slowly released from micropipettes) or is a typical characteristic of substance P responses. A number of authors have suggested that substance P may act to modulate (either sensitization or desensitization) the actions of conventional neurotransmitters, thus accounting for its slow onset of action. To settle many of these questions a specific antagonist for substance P is required.

Several groups have shown, using either immunohistochemical techniques or bioassay, that substance P is transported by axoplasmic transport (Otsuka and Konishi, 1977; Hokfelt et al., 1978; Paxinos, Emson and Cuello, 1978). Immunohistochemical studies suggest that substance P undergoing transport is sequestered in vesicles (Hokfelt, Johansson et al., 1977; Cuello, Emson et al., 1978). The rate at which substance P is transported in rat cervical vagus has been reported to be 25-50 mm/day (Gilbert and Emson, 1978). This rate is similiar to that found for the transmitter synthetic enzymes tyrosine hydroxylase and dopamine-beta-hydroxylase which are also transported in vesicles in adrenergic nerves (Brimijoin, 1972).

Experiments by Nakata et al. (1978) have demonstrated that tritiated substance P binds to a receptor site in rabbit CNS (K_d=2.74 nM). The distribution of these binding sites correlates well with the reported distribution of substance P terminals. Experiments with substance P fragments indicated that the C-terminal heptapeptide has

the highest affinity for the receptor (K_I=5.6 X 10^{-11} M). The potencies of various substance P fragments in displacing tritiated substance P binding were consistent with the reported biological activity of each fragment. Sectioning of dorsal roots leads to a supersensitivity of lumbar spinal cord interneurons to substance P (Wright and Roberts, 1978). This suggests that an increase in the number of substance P receptors may occur following loss of the major substance P input into the spinal cord.

A substance P inactivating enzyme has been identified in brain by Benuck and Marks (1975). The enzyme exhibited properties which indicated that it was a neutral proteinase. It is, however, unclear whether this enzyme is actually normally involved in the degredation of extracellular substance P, as the enzyme was isolated from the cytosolic fraction of brain homogenates. Soluble peptidases which can inactivate substance P are found in all brain areas (Marks, 1977) but none has yet been implicated in the inactivation of extracellular substance P. Thus, a strong case exists for considering substance P as an excitatory neurotransmitter in several physiological pathways, particularly the nociceptive small diameter C and/or A delta fibers.

Regulation of Substance P

Little is known regarding the factors involved in the regulation of substance P content of primary afferent neurons. Recently, it has been shown that substance P is depleted from the sensory neurons of neonatal (Gorin and Johnson, 1979; Otten et al., 1980; Ross et al., 1981) and adult (Schwartz, Pearson and Johnson, 1982) animals exposed to

antibodies to nerve growth factor (NGF). In neonates, this depletion is accompanied by neuronal degeneration (Gorin and Johnson, 1979; Otten et al., 1980; Ross et al., 1981) while in adults cell death does not occur (Schwartz et al., 1982). This suggests that sensory neurons must continually be exposed to NGF to maintain substance P levels. NGF is thought to be synthesized near the peripheral terminals of primary afferent neurons and to gain access to the cell bodies of sensory neurons by undergoing retrograde axoplasmic transport after receptor mediated pinocytosis (Hendry et al., 1974; Stockel, Schwab and Thoenen, 1975). Sectioning of the peripheral processes of sensory neurons, which would interrupt the transport of NGF, also results in depletion of substance P from the central portions of primary afferent neurons (Jessel et al., 1979). These data suggest that the maintenance of substance P levels in primary afferent neurons is dependent upon the constant exposure of the cell bodies of sensory neurons to NGF.

Nerve Growth Factor (NGF)

NGF is a protein of wide species distribution which has been reported to be essential for the development of both sensory and adrenergic neurons (Levi-Montalcini and Angeletti, 1968). The major physiologic role for NGF appears to be to regulate the synthesis of specific macromolecules by neuronal tissue (Thoenen and Barde, 1980; Mobley, Server et al., 1977). In recent years it has been demonstrated that NGF is taken up with high selectivity at the distal ends of sympathetic and sensory nerves. NGF is then transported by axoplasmic transport in a retrograde fashion to the perikaryon (Stockel, Pavavinci

and Thoenen, 1974; Hendry, Stach and Herrup, 1974; Hendry, Stockel et al., 1974; Stockel et al., 1975). The retrograde axoplasmic transport of NGF has been studied in most detail in the sympathetic nervous system. When ^{125}I-NGF is unilaterally injected into the anterior eye chamber of rats or mice, radioactivity accumulates almost exclusively in the ipsilateral superior cervical ganglia. The radioactivity which accumulates in superior cervical ganglia after retrograde axoplasmic transport is greater than 90 percent unchanged NGF as evaluated by SDS-gel-electrophoresis (Johnson, 1978; Stockel et al., 1976). Autoradiographic and histochemical studies have shown that after uptake of NGF at the distal ends of sensory and adrenergic neurons, the NGF is localized into small vessicles and tubular cisternae (Schwab and Thoenen, 1977; Schwab, 1977).

The biological effects of NGF have been characterized best in sympathetic adrenergic neurons. NGF produces a pleotrophic response in adrenergic neurons which is characterized by increases in protein and RNA content (Levi-Montalcini and Angeletti, 1968; Varon, 1975; Thoenen and Barde, 1980; Thoenen et al., 1971; Hendry, 1975). This generalized growth is preceeded by a marked increase in the activity of ornithine decarboxylase (Thoenen et al., 1979; MacDonnell et al., 1977), an enzyme whose induction is commonly associated with rapid cellular growth (Ochoa and de Haro, 1979). In addition, NGF accelerates the differentiation of sympathetic neuroblasts into mature neurons (Thoenen et al., 1971; Angeletti, Levi-Montalcini and Caramia, 1971).

Biochemically this enhanced differentiation induced by NGF may be demonstrated by measuring the activities of tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH), two enzymes involved in the synthesis of norepinephrine. NGF appears to induce, with some specificity, the activities of both TH and DBH (Angeletti et al., 1972; Thoenen, 1972; Otten et al., 1977; Schaefer, Schwab and Thoenen, 1979).

In sensory neurons, like adrenergic sympathetic neurons, NGF appears to be involved in the regulation of cell growth and differentiation (Levi-Montalcini and Angeletti, 1968). However, unlike sympathetic neurons where cell growth and differentiation may be studied by measuring the activity of enzymes such as TH and DBH, no specific biochemical markers exist for sensory neurons. Recently, substance P has been suggested to be a marker of sensory neurons. However, its usefulness may be limited because it is not found in all sensory neurons nor is it found only in sensory neurons (Kessler and Black, 1980).

As in sympathetic neurons, NGF induces the formation of nerve fibers in cultured sensory neurons (Levi-Montalcini et al., 1968; Greene, 1977). These nerve fibers always grow towards higher concentrations of NGF in vitro (Charlwood, Lamont and Banks, 1972; Letourneau, 1978). Hence it clear that both sympathetic and sensory neurons may be affected by exposure to exogenous NGF. The pharmacology of endogenous NGF has only recently been investigated by the use of antibodies to NGF.

It has been suggested that NGF may be required for the normal development of both adrenergic and sensory neurons. Data supporting this postulate comes from studies in which transplacental transfer (Johnson et al., 1980; Gorin and Johnson, 1979) or direct embryonic injection (Levi-Montalcini et al., 1980) of NGF antibodies were shown to destroy the majority of adrenergic and sensory neurons in rats.

Effect of Capsaicin on Substance P

In 1978 Jessell, Iversen and Cuello investigated the effects of capsaicin desensitization on substance P in primary afferent neurons. Male rats were treated with capsaicin for five consecutive days (50, 100, 200, 200, 400 mg/kg s.c.). Substance P, opiate receptor binding, and glutamic acid decarboxylase (GAD) activity were measured in dorsal spinal cord homogenates 10 days after the first capsaicin injection. Substance P levels were found to be decreased by 48 percent while opiate receptor binding and GAD activity remained unchanged. Since opiate receptors are located on the terminals of primary sensory afferent neurons which terminate in the dosal horn of the spinal cord (La Motte et al., 1976), these results indicated that capsaicin probably selectively inhibited the synthesis, storage, or metabolism of substance P without causing degeneration of substance P containing neurons. The fact that GAD activity remained unchanged suggested that capsaicin acted selectively on primary afferent neurons and not on GABAergic interneurons.

The effect that capsaicin has on selected neural tissues of the rat following acute administration was then investigated. Capsaicin was found to produce a Ca^{++} dependent release of substance P from

superfused spinal cord slices in vitro (Theriault, Otsuka and Jessell, 1979; Gamse, Holzer and Lembeck, 1979). In contrast, capsaicin did not promote substance P release from slices of hypothalamus or substantia nigra (Gamse et al., 1979). This suggested that the thermoregulatory effects of capsaicin do not involve substance P release. The results of Gamse et al. (1979) tend to indicate that substance P containing neurons in the spinal cord are selectively sensitive to the actions of capsaicin. The mechanism of this specificity is unknown.

The effect of capsaicin administration to neonatal rats has been investigated by several authors. Nagy et al. (1980) administered capsaicin (50 mg/kg s.c.) to rats on the second day of life. After 12 weeks substance P levels in dorsal horn of the spinal cord were decreased by almost 50 percent while levels in the hypothalamus, striatum, and substantia nigra remained unchanged. Opiate receptor density, as measured by ^{3}H-naloxone binding, was decreased in the dorsal spinal cord. GAD and choline acetyltransferase activities in dorsal spinal cord were found to be unchanged. It was concluded that capaicin-induced selective degeneration of substance P containing neurons in sensory ganglia following neonatal administration. Histological evidence supporting capsaicin-induced degeneration of substance P neurons was presented by Nagy and Vincent (in press). Histological analysis of the dorsal spinal cords of two day old rats 6 hours after capsaicin administration (50 mg.kg s.c.) revealed many degenerating boutons and unmyelinated axons which were engulfed by glial elements. The mechanism of the age-related effects of capsaicin on sensory neurons is not known.

STATEMENT OF THE PROBLEM

Capsaicin, the principal pungent and irritating component of hot peppers, produces desensitization to chemogenic nociceptive stimuli following parenteral administration. In addition, capsaicin is capable of depleting the putative nociceptive peptide neurotransmitter, substance P, from primary afferent neurons. Thus, capsaicin is a useful pharmacologic tool for investigating sensory neuron function. Moreover, capsaicin is ingested as a herbal medicine and dietary condiment throughout the world in quantities which often exceed 70 mg/day. Hence, capsaicin also represents a potential environmental hazard. The biochemical mechanisms by which capsaicin alters sensory function and depletes substance P from primary afferent neurons are currently unknown and were the subject of this investigation. The specific aims of this research were as follows:

a. To characterize in detail the specific sensory modalities affected by capsaicin.
b. To gain insight as to the biochemical mechanism by which capsaicin alters sensory function.
c. To determine the relationship between substance P depletion by capsaicin and the production of antinociception.
d. To determine the biochemical mechanism by which capsaicin depletes substance P from primary afferent neurons.

METHODS AND MATERIALS

Drugs and Chemicals

Zingerone was purchased from Pfaultz and Baurer, Inc. Stamford, CN. Tyr^8-substance P and substance P were obtained from Penninsula Laboratories, Palo Alto, CA and Vega Biochemicals, Tucson, AZ, respectively. L-(2,3,4,5-^{3}H)-proline, L-(2,3-^{3}H-(N))-tryptophan and carrier free tritium gas were puchased from New England Nuclear, Boston, MA. Goat anti-rabbit immunoglobulin precipitating complex was purchased from immunonuclear Corporation, Stillwater, MN. Morphine sulfate and ethylmorphine were obtained from Merck Chemical Co., Rahway, NJ. Nonylvanylamide, vanillylamide hydrochloride and pelargonaldehyde were purchased from Fluka Chemical Corporation, Hauppauge, NY. SKF 525A was a generous gift of Smith, Kline and French, Philadelphia, PA. Pentobarbital was obtained from Abbott Laboratories, Chicago, IL. Radioiodine was purchased from Amersham, Arlington Heights, IL and Enzymobeads^R were obtained from Bio Rad Laboratories, Richmond, CA. All other drugs or chemical were purchased from Sigma Chemical Co., St. Lous, MO.

Effect of Capsaicin on Peripheral Sensory Neurons

The actions of capsaicin on peripheral sensory function were investigated in Hartley guinea pigs of either sex (300-400 g) which were treated with a single dose of capsaicin (50 mg/kg s.c.) or capsaicin vehicle (1.0 ml/kg s.c.). Capsaicin vehicle consisted of 10

percent ethanol: 10 percent Tween 80: 80 percent 0.09 percent saline. Immediately after capsaicin injection, all animals were treated with aerosol isoproterenol (0.75 percent) and placed in an oxygenated chamber to prevent death due to bronchoconstriction. Three days after capsaicin treatment peripheral sensory function in each animal was assessed by determining the response of each animal to a series of peripheral sensory stimuli. A separate group of animals (N=5) were treated with morphine sulfate 30 minutes prior to sensory testing and served as positive controls.

One day after peripheral sensory testing animals were killed by decapitation and dorsal root ganglia C_4-T_1, dorsal spinal cord C_4-T_1, ventral spinal cord C_4-T_1, hypothalamus, and corpus striatum were removed for determination of substance P content by radioimmunoassay (RIA). In addition, the amino acid content of dorsal spinal cord was determined. Tissues were stored at -80 ^{o}C until time of assay.

Techniques for Assessing Sensory Function

Hot Plate Test

The hot plate test measures the ability of animals to sense thermal (heat) nociceptive stimuli. The test was conducted by placing an animal on a glass warming tray which was heated to 55^{o}C. Temperature of the tray was regulated by connecting the tray to a rheostat. The latency for escape of each animal from the plate was then measured.

Skin Flinch Test

The skin flinch test, like the hot plate test, is a measure of thermal (heat) nociception. To conduct the test the fur on the back of each animal was first removed by shaving. A dipilatory agent (Neet^{R}) was then applied to the back of each animal to remove the remaining fur and the exposed skin was thoroughly washed to remove any remaining depilatory agent. The skin flich test was then conducted by directing a 500 watt projector lamp at the exposed skin from a distance of approximately 2 cm. The latency to a skin flinch, or perniculous reflex, in response to the thermal stimulation was then measured.

Heat Probe Test

The heat probe test is designed to be a measure of the ability to sense non-nociceptive thermal (heat) stimuli. To conduct the test the fur was removed from the back of each animal as descibed above. A hot soldering iron was then brought to a distance of 1 cm from the exposed skin. Induction of a skin flich, or perniculous reflex, within 5 seconds was considered a positive response.

Cold Probe Test

The cold probe test is designed to be a measure of the ability to sense non-nociceptive cold stimuli. To conduct the test the fur was removed from the back of each animal as descibed above. A piece of dry-ice was then brought to a distance of 1 cm from the exposed skin. Induction of a skin flich, or perniculous reflex, within 5 seconds was considered a positive response.

Air Puff Test

The air puff test measures the ability of animals to sense non-nociceptive mechanical stimuli. To conduct the test, the fur was removed from the back of each animal as descibed above. The exposed skin was then gently stimulated with a jet of air which eminated from a Pasteur pipette which was connected to a compressed air source. Induction of a skin flich, or perniculous reflex, within 5 seconds was considered a positive response.

Zingerone Test

The zingerone test was a measure of the ability of each animal to sense nociceptive chemogenic stimuli. To conduct the test 50 ul of a solution containing 1 percent zingerone (w/v) in 0.09 percent saline was placed in one eye of each animal. The duration for which animals violently wipe and scratch the eye was then measured.

Pressure Test

The pressure test is thought to be a measure of the ability to sense nociceptive mechanical distortion. To conduct the test, pressure was applied to a front foot of each animal with a sealed inverted 3 ml plastic syringe. The amount of pressure which was applied to the foot of each animal was slowly increased until the animal responded with either vocalization or an escape response. The quantity of pressure applied to the animals foot was then determined by measuring the extent to which the air trapped within the sealed syringe was compressed.

Anesthesiometer

The anesthesiometer, like the air puff test, measures the ability of animals to sense non-nociceptive mechanical stimuli. The anesthesiometer consists of a series of 10 nylon probes ranging in diameter from 0.2 to 0.5 mm. The test is conducted by placing the probes on the bare back skin of each animal beginning with the smallest. Enough pressure is applied with each probe to cause the probe to bend. The smallest diameter probe exerts the least pressure on the skin prior to bending. The number of the smallest probe which is capable of producing a skin flinch, or perniculous reflex, when applied to the animal's skin is recorded.

Cold Plate Test

The cold plate test measures the ability of animals to sense nociceptive cold stimuli. The test is conducted by placing an animal on a flat block of dry-ice. The latency for escape from the dry-ice is then measured.

Substance P Assay

RIA for substance P was conducted Stephen Buck using antisera which was generously provided to Dr. H.I. Yamamura by Dr. Marvin R. Brown of The Salk Institute. Tissues were weighed while frozen and homogenized in 10-20 volumes of 2 M acetic acid with a Tekmar polytron at the maximum setting for 15 seconds. Homogenates were then centrifuged at 10,000 X g for 15 minutes. The pellet was discarded and

the supernatant was frozen at -80 ^{o}C and lyophilized to dryness. Extraction of substance P from tissue was estimated to be 90.3 $\pm$ 1.1 percent using ^{125}I-Tyr^{8}-substance P as standard. The lyophilized tissue pellet was then resuspended in 1.0 ml of phosphate buffered normal saline. Approximately 100 ul of each resuspended tissue pellet was then subjected to equilibrium RIA using ^{125}I-Tyr^{8}-substance P prepared form Tyr^{8}-substance P by the chloramine T method (Mroz and Leeman, 1979) as tracer. Specific activity of tracer was approximately 2200 Ci/mmole. A 1:50,000 dilution of antisera was employed. This yielded a maximum binding of 31.5 percent. Sensitivity of the RIA for substance P was approximately 7 fmole (Fig. 4) in 100 ul of sample and the K_a of the antisera for authentic substance P standard was 1.2 X 10^{-12} M. Cross-reactivity for displacement of tracer by other neuropeptides is shown in Table 1. Physalaemin and eledoisin, two non-mamalian peptides which are similiar in structure to substance P, required 3 orders of magnitude greater concentrations to produced detectable displacement of tracer while the mamalian peptide somatostatin required 4 orders of magnitude greater concentrations to produced detectable displacement of tracer. All other neuropeptides tested were devoid of the ability to displace tracer within 6 orders of magnitude.

Measurement of Dorsal Spinal Cord Amino Acid Content

Portions of guinea pig dorsal spinal cord were sonicated (Branson Sonifier^{R} model 200) for 10 seconds in 3.5 percent sulfosalicylic acid containing 0.25 mM gamma-amino-n-butyric acid as

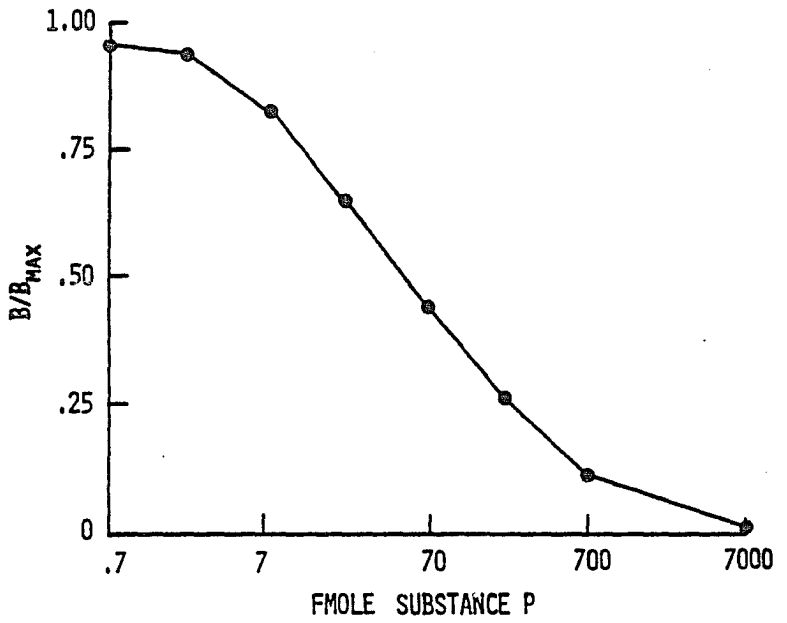


Figure 4. Standard curve for substance P RIA.-- Antisera dilution was 1:50,000, maximum binding equaled 35 percent, $r_{(7\text{-}700\ fmole)}$ = -0.99.

Table 1. Displacement of ^{125}I-Tyr^{8}-substance P by neuropeptides.

Peptide	Amount (fmole)	% Displacement of binding[a]
Substance P	7	11.5
Physlaemin	7000	25.8
Eledoisin	7000	14.5
Somatostatin	70000	17.9
Bombesin	7000000	23.7
Bradykinin	7000000	< 10
VIP	7000000	< 10
Pentagastrin	7000000	< 10
CCK (27-33)	7000000	< 10
Neurotensin	7000000	46.0
TRH	7000000	14.1
Angiotensin II	7000000	< 10
Alpha Endorphin	7000000	12.0
Beta Endorphin	7000000	< 10
Gamma Endorphin	7000000	< 10
Leu^{5}-beta-endorphin	7000000	< 10
Met-enkephalin	7000000	< 10
Dynorphin (1-13)	7000000	< 10
Des-tyr-gamma-endorphin	3500000	< 10

[a] 10 percent displacement of tracer was considered the minimum requirement for cross reactivity.

internal standard. The homogenates were centrifuged in a Beckman model 11 microcentrifuge and the supernatants were mixed with one half volume of 0.3 N lithium hydroxide. Aliquots of this solution were analyzed by conventional lithium citrate methodology on a Beckman model 118C amino acid analyzer (Huxtable and Laird, 1978).

Temporal Relationship Between Substance P Depletion and Antinociception

The temporal relationship between substance P depletion and the induction of antinociception by capsaicin was investigated in Hartley guinea pigs which were treated with a single dose of capsaicin (50 mg/kg s.c.) or capsaicin vehicle (1.0 ml/kg s.c.) as described above. Escape latency to a 55 ^{o}C hot plate was determined immediately prior to, 1 day after and 4 days after capsaicin injection. Immediately after sensory testing, animals were killed and substance P content of dorsal root ganglia C_4-T_1 was determined using the RIA for substance P described above.

Dose-response for Depletion of Substance P

The dose-response relationship for depletion of substance P from primary afferent neurons by capsaicin was investigated in Hartley guinea pigs (350-450 g). Guinea pigs were treated with capsaicin (2-50 mg/kg s.c.) and substance P content of dorsal root ganglia C_4-T_1 was determined 1 day and 4 days after capsaicin treatment as described previously.

Dose-response for Capsaicin-induced Antinociception

The dose response relationship for capsaicin-induced chemogenic antinociception was determined in Hartley guinea pigs 3 days after treatment with capsaicin (2-50 mg/kg s.c.). To quantitatively assess chemogenic nociception, 50 ul of zingerone was placed in one eye of each animal. The duration for which animals responded to ocular zingerone was then measured.

Effect of Capsaicin on Substance P Synthesis

Hartley guinea pigs were treated with a single dose of capsaicin (50 mg/kg s.c.) or capsaicin vehicle (1.0 ml/kg s.c.) as described above. Three and one half days (84 hours) after capsaicin treatment all animals received a single injection of L-(2,3,4,5-^{3}H)-proline (50 uCi i.p.; specific activity = 136 Ci/mmole). Capsaicin and vehicle-treated animals (5/group) were killed 12 hours and 24 hours after injection of ^{3}H-proline. Dorsal root ganglia C_4-T_5 were collected from each animal and incorporation of ^{3}H-proline into substance P was determined. Dorsal root ganglia were weighed while frozen and homogenized in 10-20 volumes of 2 M acetic acid with a Tekmar polytron at the maximum setting for 15 seconds. Homogenates were then centrifuged at 10,000 X g for 15 minutes. The pellet was discarded and the supernatant was frozen at -80 ^{o}C and lyophilized to dryness. The pellet was then resuspended in 1.0 ml of phosphate buffer normal saline. Ten ul of substance P antisera (1:100 dilution) was then added to each resuspended pellet and the solutions were allowed to

equilibrate for 18 hours. One ml of goat anti-rabbit immunoglobulin precipitating complex was then added to each assay tube. The solutions were allowed to equilibrate for an additional 18 hours and were centrifuged at approximately 1500 X g for 15 minutes at 4 ^{o}C in a Sorval GLC centrifuge. The supernatant was subsequently discarded. Five hundred ng of substance P tracer and 0.5 ml of 0.1 M potasium phosphate buffer, pH 2.2, were then added to each tube. To dissociate substance P from substance P antibodies the mixtures were placed in a boiling water bath for 10 minutes. The solutions were centrifuged at approximately 2,000 X g in a Beckman microfuge (model 11). The substance P content of each sample was then isolated by high pressure liquid chromatography (HPLC) and the quantity of tritium incorporated into substance P was determined by liquid scintillation counting in a Beckman LS8100 LSC after addition of 15 ml of Betaphase^{R}.

Separation of Substance P by HPLC

Separation of substance P by HPLC was accomplished using a Waters model P/N 27324 S/N C_{18} reverse phase column. Mobile phase was 0.1 M potasium phosphate buffer, pH 2.2, containing 25 percent acetonitrile. All solvents were filtered by means of a Millipore 0.46 um filter and degassed prior to use. Flow rate was 1.0 ml minute and substance P was detected using a Waters model 441 UV detector monitoring at 214 nm. Under these conditions the retention time for substance P was approximately 14 minutes (Fig. 10).

Effect of Capsaicin on Total Protein Synthesis

The effect of capsaicin on the rate of total protein synthesis in dorsal root ganglia was investigated in Hartley guinea pigs which had been treated with a single dose of capsaicin (50 mg/kg s.c.) or vehicle (1.0 ml/kg s.c.). Three and one half days (84 hours) after capsaicin treatment all animals received a single injection of L-(2,3-^{3}H-(N))-tryptophan (0.5 uCi i.p.; specific activity=10 Ci/mmole). Capsaicin and vehicle-treated animals (5/group) were killed 6 hours and 12 hours after injection of ^{3}H-tryptophan. Dorsal root ganglia C_4-T_1 were collected from each animal and incorporation of ^{3}H-tryptophan into total protein was determined. Dorsal root ganglia were weighed while frozen and homogenized in 10-20 volumes of 0.05 M Tris-HCL buffer, pH 7.4, using a Tekmar polytron set at the maximum speed for 15 seconds. Protein was precipitated by the addition of 2.0 ml of ice-cold ethanol followed by centrifugation at 1,500 X g in a Sorval GLC centrifuge. Radioactivity not incorporated into protein was extracted from the protein pellet with the following series of solvents: Chloroform/ethanol (1:3), 100 percent ethanol, 0.01 M HCl, methanol/ether (3:1) and acetone. Pellets were washed with each solvent until no radioactivity was detected in the wash. The resulting protein pellets were digested with 1 N sodium hydroxide, neutralized with 1 N perchloric acid and tritium incorporated into protein was determined by liquid scintillation counting.

Structure Activity Relationships for Capsaicinoids

Synthesis of Dihydrocapsaicin

Capsaicin was suspended in methanol within a closed hydrogen filled flask which was attached to a hydrogen filled manometer. One ml of 5 percent palladium on charcoal (200 mg/ml) which had been previously equilibrated with hydrogen was added to the capsaicin containing flask by syringe through a Teflon septum. The suspension was stirred constantly and uptake of hydrogen was measured (Fig. 5). The reaction was considered complete when hydrogen uptake ceased. The suspension was then centrifuged at 1,500 X g for 20 minutes in a Sorval GLC centrifuge to remove catalyst and dihydrocapsaicin was recrystalized from n-hexane. Contamination of dihydrocapsaicin with capsaicin was estimated to be less than 5 percent by melting point analysis and direct probe chemical ionization mass-spectroscopy on a Finnigan model 3300 mass spectrometer equipped with an INCOS 2061 data system (Fig. 6).

Radiolabelled dihydrocapsaicin was similiarly prepared by the addition of carrier free tritium gas (5 Ci) to the reaction flask through a specially prepared glass side arm. Specific activity of ^{3}H-dihydrocapsaicin was 674 mCi/mmole.

Effect of Capsaicin Analogs on Peripheral Sensory Function and Primary Afferent Neuron Substance P Content

Hartley guinea pigs of either sex received 6 consecutive daily doses of capsaicin, nonylvanylamide, dihydrocapsaicin, vanillylamide hydrochloride, pelargonaldehyde (0.0325, 0.1625, 0.325, 0.65, 0.65,

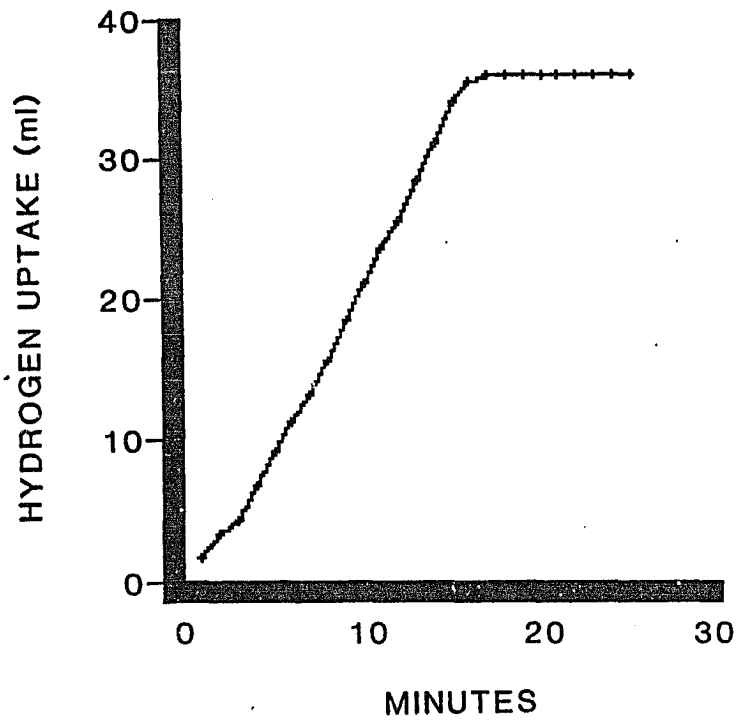


Figure 5. Uptake of hydrogen gas by reaction during hydrogenation of capsaicin to form dihydrocapsaicin.

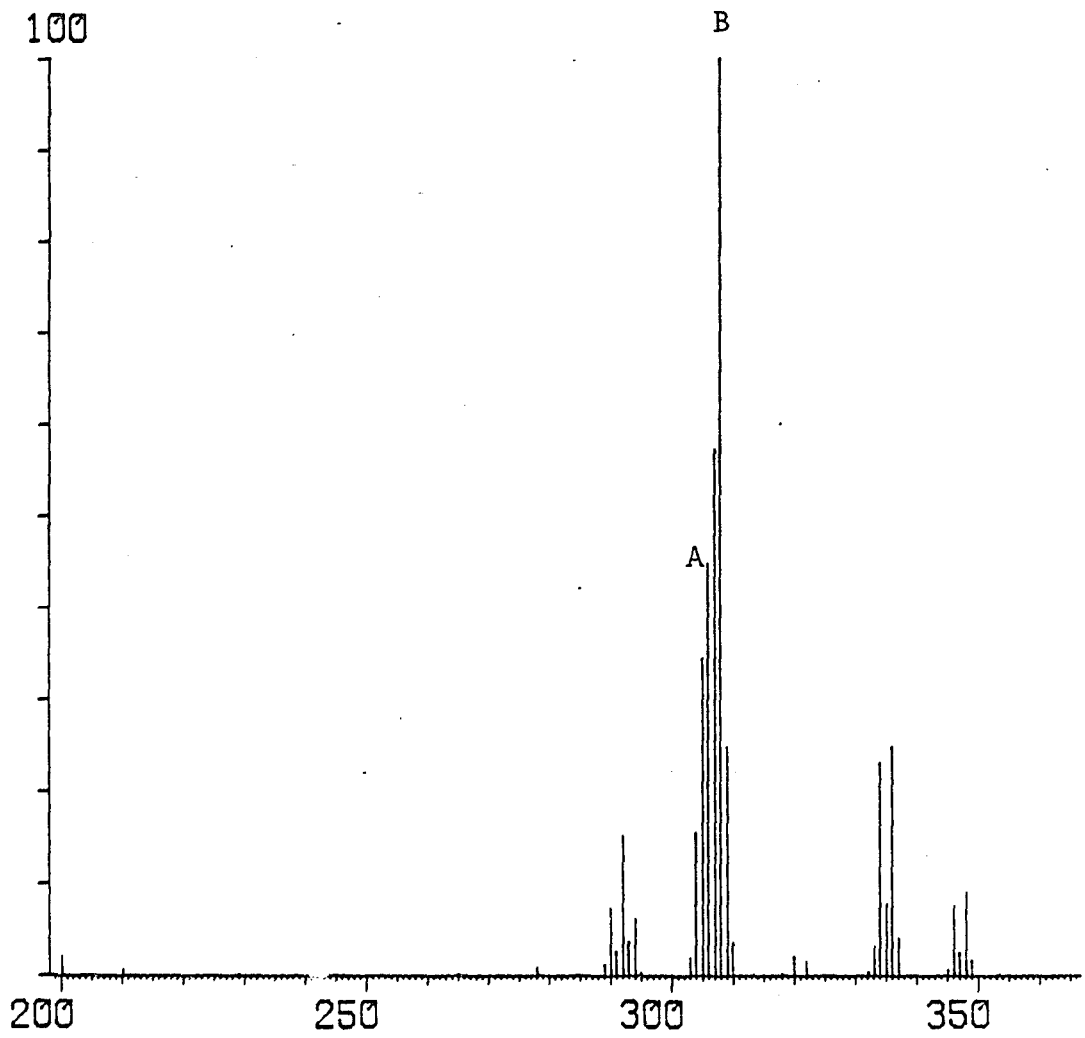


Figure 6. Chemical ionization mass spectrogram of dihydrocapsaicin produced by hydrogenating capsaicin.-- A-mass peak for capsaicin, B-mass peak for dihydrocapsaicin.

0.65, 0.65 mmole/kg s.c.; 1.0 ml/kg) or vehicle (Fig. 7). Three days after capsaicin treatment peripheral sensory function in each animal was assessed by determining the response of each animal to a series of peripheral sensory stimuli as described previously.

One day after peripheral sensory testing animals were killed by decapitation and dorsal root ganglia C_4-T_1, dorsal spinal cord C_4-T_1, ventral spinal cord C_4-T_1, hypothalamus, and corpus striatum were removed for determination of substance P content by RIA. Tissues were stored at -80 ^{o}C until time of assay.

Pharmacology of Dihydrocapsaicin

Thermoregulation

The dose-response relationship for alterations in thermoregulation induced by capsaicin and dihydrocapsaicin was investigated in female Sprague-Dawley rats (200-250 g). Animals were placed in a specially designed wire mesh restraint apparatus and Yellow Springs Instruments Co. thermistor probes (YSI 401) were inserted into the rectum to a depth of 6 cm. Thermistors were connected to a Tele-Thermometer (YSI 41TUC) for temperature measurement. Animals were allowed to acclimate to restraint for at least 4 hours prior to further experimentation. Dihydrocapsaicin and capsaicin were then administered in doses ranging from 0.05-10 mg/kg s.c. and body temperatures were measured at 15 minutes intervals for a minimum of 6 hours.

The effect of previous exposure to capsaicin or dihydrocapsaicin on the thermoregulatory response to subsequent exposures was investigated

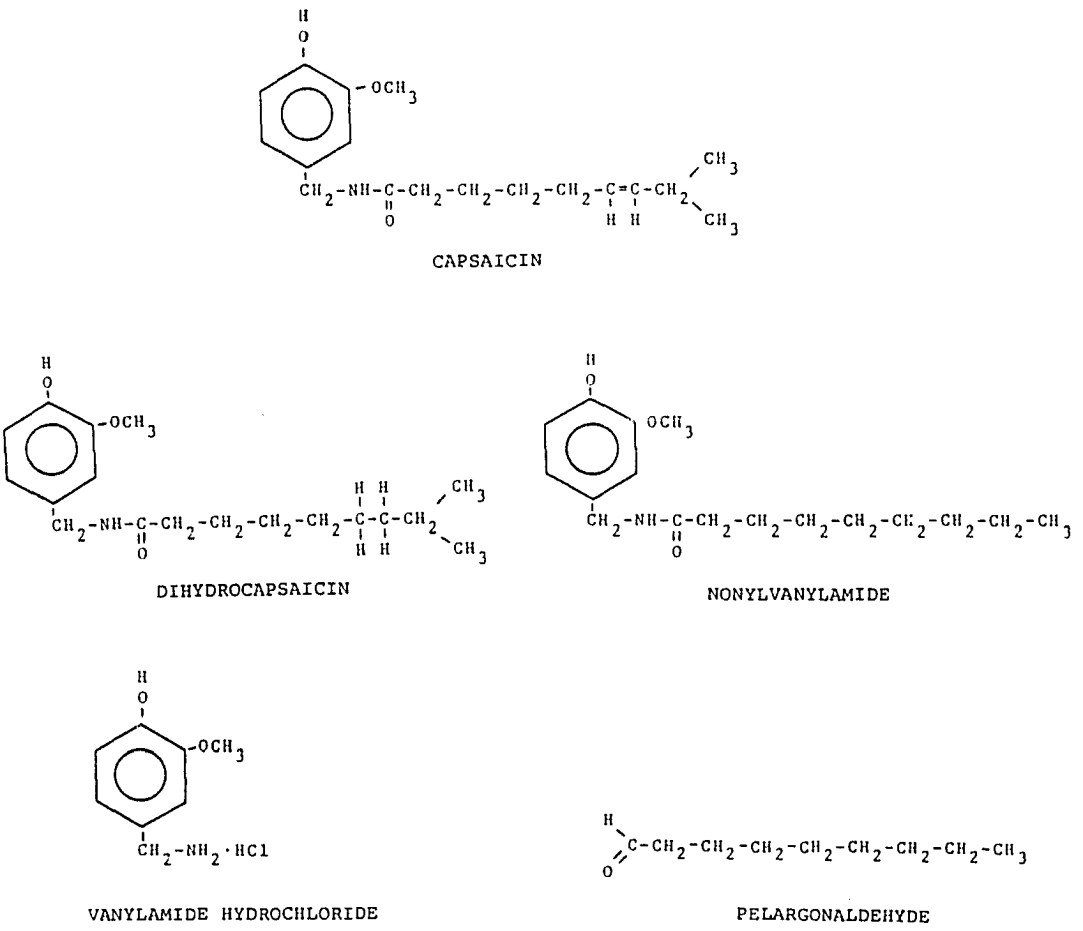


Figure 7. Structures of capsaicin analogs.

in rats treated according to the protocol decribed in Table 2. Cross-tolerance was investigated by determining the thermoregulatory response when dihydrocapsaicin (2.0 mg/kg s.c.) was administered to rats previously treated with 6 consecutive daily doses of capsaicin (10, 50, 100, 100, 100, 100 mg/kg s.c.). In addition, the thermoregulatory response to capsaicin (2.0 mg/kg s.c.) was studied in rats pretreated with 6 consecutive daily doses of dihydrocapsaicin (10, 50, 100, 100, 100, 100 mg/kg s.c.).

Nociception and Substance P

The effects of capsaicin and dihydrocapsaicin on primary afferent function were determined in rats 1 day and 5 days following treatment with 6 consecutive daily doses of vehicle (10% ethanol: 10% Tween 80: 80% saline), capsaicin or dihydrocapsaicin (10, 50, 100, 100, 100, 100 mg/kg s.c.; 1.0 ml/kg s.c.). Chemogenic nociception was assessed by determining the response to topical application of 1% zingerone (w/v) to the cornea. Thermal antinociception was assessed by determining tail-flick latencies in response to a 500 watt projector lamp directed at the tail from a distance of 6 cm. Animals were killed 6 days after the last capsaicin dose and substance P content of the hypothalamus, corpus striatum, dorsal root ganglia, dorsal spinal cord and ventral spinal cord was determined by the substance P RIA previously described.

Table 2. Protocol for investigating the effects of repeated administration of capsaicin or dihydrocapsaicin on thermoregulatory response.

Day	Capsaicin or dihydrocapsaicin Dose (mg/kg s.c.)	Temperature Measured
1	2	+
2	2	+
3	2	+
4	2	+
5	10	-
6	50	-
7	100	-
8	100	-
9	100	-
10	100	-
11	0	-
12	2	+

+ thermoregulatory response measured
- thermoregulatory response not measured

Biotransformation of Capsaicin and Dihydrocapsaicin

Animals

Adult female Sprague-Dawley rats weighing approximately 200 g were used in all biotransformation experiments. Animals were housed in hanging metal cages with free access to food and water. Temperature was maintained at 20-23^{o}C and light/dark cycles were alternated at 12 hour intervals. All animals were killed by decapitation.

Microsome Preparation

Immediately following decapitation, the liver of each animal was perfused through the hepatic portal vein with ice-cold 0.05 M Tris-HCl buffer, pH 7.4, containing 1.15 percent KCl (Tris-KCl buffer). Livers were removed, cut into small pieces and homogenized with a Potter-Elvehjem glass-Teflon homogenizer in 3 volumes of 0.05 M Tris-KCl buffer. The homogenate was centrifuged at 15,000 X g for 20 minutes at 4^{o}C in a Sorval (model RC2-B) centrifuge. The supernatant was removed and centrifuged at 100,000 X g for 50 minutes in a Beckman L8-55 ultracentrifuge. The resulting microsomal pellet was washed by resuspension in 0.05 M Tris-KCl buffer followed by recentrifugation at 100,000 X g for 50 minutes. The washed microsomal pellet was suspended in 0.05 M Tris-KCl buffer. A portion of microsomes was suspended in 0.1 M phosphate buffer, pH 7.4, for determination of spectral interactions. All steps were carried out at 4^{o}C. Protein concentration was determined by the Biuret method (Gornall, Bardawill and David, 1949).

Spectral Interactions

Phospate buffered rat liver microsomes were diluted to a protein concentration of 2.0 mg/ml. Spectra were measured at room temperature in 1 cm with a Beckman Acta CIII dual beam spectrophotometer. Difference spectra were obtained by the addition of microliter quantities of capsaicin solutions prepared in distilled water to the sample cuvette which contained 800 ul of microsomal suspension. The reference cuvette received an equal volume of distilled water. Difference spectra were recorded between 350 and 500 nm. The extent of spectral change was considered to be the absorbtion difference between wavelengths of minimum and maximum absorbance. The K_s for spectral interaction was calculated by Eadie-Hofstee analysis (Gillette, 1972).

Microsomal Demethylase Assay

Capsaicin (1 mM) and/or ethylmorphine were incubated for 15, 30 and 60 minutes at 37^{o}C with microsomal protein (2 mg) and NADPH generating system consisting of NADP (0.8 mg), glucose-6-phosphate (3 mg) and glucose-6-phosphate dehydrogenase (10 units) in a final volume of 2.0 ml. The NADPH generating system was omitted in control incubations. All determinations were conducted in duplicate. Formaldehyde produced as a result of demethylation was measured by the method of Nash (1953) as modified by Cochin and Axelrod (1959).

Inhibition Studies

Ethylmorphine (0.25, 0.5, 1.0 and 2.0 mM) was incubated at 37^{o}C with microsomal protein (2 mg) and capsaicin (0, 25 and 50 uM) or SKF

525A (25 uM) in a total volume of 2.0 ml. After 15 minutes, formaldehyde production was assessed by the method of Nash (1953) as modified by Cochin and Axelrod (1959) and data were analyzed by Eadie-Hofstee analysis (Gillette, 1972).

Pentobarbital Sleep Time

Pentobarbital sleep time was measured in female Sprague-Dawley rats which weighed approximately 200 g. Sodium pentobarbital (30 mg/kg i.p.) was administered to animals 6 hours after treatment with capsaicin (10 mg/kg s.c.) or vehicle. Time to regain righting reflex was measured. Data were analyzed by Student's t-test.

Dihydrocapsaicin Binding to Microsomal Protein

Dihydrocapsaicin (1 mM) containing 1 uCi of ^{3}H-dihydrocapsaicin was incubated with NADPH generating system and microsomal protein (2 mg) for 15, 30, 60 and 90 minutes at 37 ^{o}C in a total volume of 2.0 ml. Nonenzymatic background binding was determined by use of microsomes boiled for 5 minutes prior to incubation or by omitting the NADPH generating system from the incubations. These treatments gave essentially the same level for background binding. Incubations were terminated by the addition of 2.0 ml of ice-cold ethanol. Unbound radioactivity was then extracted with the following series of solvents; Chloroform/ethanol (1:3), 100 percent ethanol, 0.01 M HCl, methanol/ether (3:1) and acetone. This solvent series extracts 100 percent of ^{3}H-dihydrocapsaicin added to liver, brain and spinal cord homogenates. The resulting protein pellet was digested in 1 N sodium

hydroxide. After neutralization with 1 N perchloric acid the tritium content was determined by liquid scintillation counting. Protein content of the digestate was determined by the Biuret method (Gornall et al., 1949).

Metabolites of Capsaicin and Dihydrocapsaicin

Capsaicin (1 mM) or dihydrocapsaicin (1 mM) containing 1 uCi of ^{3}H-dihydrocapsaicin were incubated with microsomal protein (2 mg) with and without NADPH generating system for 15 minutes at 37^{o}C in a total volume of 2.0 ml. Upon completion, incubations were frozen immediately in a dry-ice methanol bath and lyophilized to dryness. The lyophilizate was extracted with 500 ul of methanol and an aliquot of the extract was applied to LK5D (80 A) silica gel thin layer chromatography plates (Whatman, Inc., Clifton, NJ). Plates were eluted with ethylacetate: acetic acid (9:1), dried, and phenols were visualized by spraying the plates with 1 percent (w/v) 2,6-dichloro-quinone-4-chloroimide (Gibb's reagent) in methanol followed by exposure to ammonia vapor for 60 seconds. Plates containing radioativity were subsequently scored in 0.5 cm increments and scraped for liquid scintillation counting.

Covalent Binding of Dihydrocapsaicin In Vivo

Female Sprague-Dawley rats (200 g) received a single dose of dihydrocapsaicin (10 mg/kg s.c.) containing 5 uCi of ^{3}H-dihydrocapsaicin. Liver, brain and spinal cord were removed 3, 6, 12 and 24 hours after treatment. Tissues were homogenized in 0.05 M

Tris-KCl buffer. An aliquot of each homogenate was assayed for total radioactivity by scintillation counting following digestion with 1 N sodium hydroxide and neutralization with 1 N perchloric acid. Bound radioactivity was then measured in an aliquot of each homogenate. Unbound radioactivity was extracted with the following series of solvents; Chloroform/ethanol (1:3), 100 percent ethanol, 0.01 M HCl, methanol/ether (3:1) and acetone. The resulting protein pellet was digested in 1 N sodium hydroxide, neutralized with 1 N perchloric acid and tritium content was determined by liquid scintillation counting. Protein content of the digestate was determined by the Biuret method (Gornall et al., 1949). The remainder of each homogenate was centrifuged at 10,000 X g for 20 minutes. The supernatant (S-9 fraction) which contained cytosolic and microsomal protein was assayed for bound radioactivity as described above.

Site of Action for Dihydrocapsaicin-induced Antinociception

Effect of Local Administration of Dihydrocapsaicin

Antinociception. To investigate the site at which dihydrocapsaicin acts to produce antinociception, one front footpad of Hartley guinea pigs was injected with ^{3}H-dihydrocapsaicin (8 ug; 18 uCi) in a total volume of 20 ul. All injections were performed with animals under light ether anesthesia. The contralateral footpad was injected with an equal volume of vehicle (10% ethanol: 10% TWEEN 80: 80% saline). The pH of the ^{3}H-dihydrocapsaicin solution was identical to that of the vehicle, thereby ruling out possible pH effects. At various times after ^{3}H-dihydrocapsaicin treatment (2 hours - 10 days),

the response of each foot to a hot probe was assessed. To determine the ability to sense a local thermal stimulus, each foot was placed in contact with a steel probe heated to 100°C. Feet were unrestrained and the latency to withdrawal of vehicle treated feet from the hot probe never exceeded 1 second. Contact with the probe by capsaicinoid-treated feet was not allowed to exceed 3 seconds to prevent tissue damage. Contact with the probe for the 3 second limit was interpreted as loss of thermal sensation. Immediately after exposure to the hot probe, animals were killed.

Dorsal Root Ganglia Substance P Content. Substance P content were determined separately in ipsilateral and contralateral dorsal root ganglia C_4-T_1 by the method previously described.

Non-specific Effects of Dihydrocapsaicin at Dorsal Root Ganglia. Levels of ^{3}H-dihydrocapsaicin equivalents were determined in both ipsilateral and contralateral dorsal root ganglia C_4-T_1. Ipsilateral and contralateral dorsal root ganglia C_4-T_1 were homogenized separately in 1.5 ml of 2 M acetic acid. A 200 ul aliquot of each homogenate was removed and tritium content was determined by liquid scintillation counting.

Free and Bound Dihydrocapsaicin in Skin. Skin from ^{3}H-dihydrocapsaicin treated feet and contralateral vehicle injected feet was collected and assayed for total and unextractable radioactivity. Skin was frozen in liquid nitrogen and pulverized. The resulting powder was assayed for total radioactivity by liquid scintillation counting following digestion with 1 N sodium hydroxide.

Unextractable radioactivity was measured after extracting tissue with the following series of solvents; 100 percent ethanol, chloroform/ethanol (1:3), 100 percent ethanol, 0.01 M hydrochloric acid, methanol/diethyl ether (3:1) and 100 percent acetone. This solvent series extracts 100 percent of ^{3}H-dihydrocapsaicin added to tissue homogenates. Values for unextractable, presumably covalently bound, radioactivity were normalized for protein content. Protein was assayed by the biuret method (Gornall et al., 1949).

Mechanism of Substance P Depletion

Isolation of Nerve Growth Factor (NGF)

Mouse beta-NGF was isolated from the submaxillary salivary glands of male Swiss CD-1 mice by the method of Mobley, Schenker and Shooter (1977). One hundred fifty mice were killed by cervical dislocation and the submaxillary salivary glands were removed. Tissue was stored at -20^{0}C prior to isolation of NGF. To isolate NGF, salivary glands were homogenized with a Sorval omni-mixer model 17105 at the maximum setting for 60 seconds in 3 volumes of distilled water. The homogenate was then centrifuged at 10,000 X g for 1 hour and the supernatant was dialized against 0.05 M potassium phosphate buffer, pH 6.8, for 24 hours in dialysis "sacks", which were purchased from Sigma Chemical Co., St. Louis, MO (stock # 250-9U). The dialysate was then loaded onto a 10 X 2.5 cm liquid chromatography column which was packed with CM-52 (Whatman, Inc., Clifton, NJ) and previously equilibrated with 0.02 M phosphate buffer, pH 6.8. The column was then eluted with the same buffer until absorbance of the eluate was less than 0.5 at 280 nm.

The pH of the eluate was brought to 4.0 by the addition of 1/9 volume of 0.5 M potasium phosphate buffer, pH 4.0. Sufficient solid sodium chloride was then added to the eluate to bring the salt concentration to 0.4 M. The mixture was allowed to stand for 5 minutes after which time the mixture was centrifuged at 25,000 X g for 30 minutes. The supernatant was loaded onto a second CM-52 column which had been previously equilibrated with 0.05 M sodium acetate buffer, pH 4.0, plus 0.4 M NaCl. The column was washed with 50 ml 0.05 M sodium acetate buffer, pH 4.0, followed by 100 ml 0.05 M Tris-HCl buffer, pH 9.0. NGF was the eluted from the column with 0.05 M Tris-HCl buffer, pH 9.0, plus 0.4 M NaCl. The absorbance of the NGF containing fraction was then determined at 260 and 280 nm and the quantity of NGF isolated was calculated using the extinction coefficient determined for enolase by Warburg and Christian (1942).

Iodination of NGF

Purified mouse NGF was iodinated by a solid phase lactoperoxidase method. NGF (13 ug) was added to a suspension of Enzymobeads^{R}, Na^{125}I (1 mCi) and beta-D-glucose. After a 20 minute incubation period, the suspension was applied to a Sephadex G-75 column (0.7 X 10 cm) (Bio Rad Laboratories) to separate ^{125}I-NGF from unreacted ^{125}I. The ^{125}I-NGF peak was approximately 90 percent precipitable with trichloroacetic acid and specific activity was estimated to be approximately 80 Ci/mmole.

Retrograde Axoplasmic Transport of NGF

Hartley guinea pigs received a single unilateral injection of ^{125}I-NGF (20 ul; 1 uCi) in a front footpad. At various times thereafter (4-48 hours), animals were killed and ^{125}I content of ipsilateral and contralateral dorsal root ganglia C_4-T_1 was determined by counting in a Tracor Analytic (model 1196) automatic gamma counting system. Quantity of ^{125}I-NGF transported was equal to the ^{125}I content of ipsilateral dorsal root ganglia minus the quantity in contralateral dorsal root ganglia.

Effect of Systemic Capsaicin

The effect of systemic capsaicin administration on the retrograde axonal transport of ^{125}I-NGF was assessed in guinea pigs 4 days after treatment with systemic capsaicin (50 mg/kg s.c.). Animals received a single unilateral injection of ^{125}I-NGF (20 ul; 1 uCi) in a front footpad. At various times thereafter (4-48 hours), animals were killed and ^{125}I content of ipsilateral and contralateral dorsal root ganglia C_4-T_1 was determined by gamma counting. Quantity of ^{125}I-NGF transported was equal to the ^{125}I content of ipsilateral dorsal root ganglia minus the quantity in contralateral dorsal root ganglia.

Dose-response for Inhibition of NGF Transport

The dose-response relationship for capsaicin-induced inhibition of the retrograde axoplasmic transport of NGF was investigated in guinea pigs which received a unilateral injection of ^{125}I-NGF in a front footpad immediately after, and 3 days after treatment with

capsaicin (6.25-50 mg/kg s.c.). Animals were killed 24 hours after injection of ^{125}I-NGF and ^{125}I content of dorsal root ganglia was assessed.

Effect of Local Capsaicin

The effect of locally administered capsaicin on retrograde axoplasmic transport was assessed in guinea pigs 4 days after local injection of capsaicin (8 ug; 20 ul) or vehicle into a front footpad. ^{125}I-NGF was injected at the site of local capsaicin administration. Animals were killed 24 hours after treatment with ^{125}I-NGF and quantity transported was assessed as previously described.

Effect of NGF Supplementation on Substance P Depletion

Hartley guinea pigs (300 g) were treated with a single dose of capsaicin (10 mg/kg s.c.). Immediately after capsaicin administration, animals were treated with mouse NGF (1.0 mg/kg/day i.p.). Administration of NGF was subsequently repeated daily for 3 days. Four days after capsaicin treatment, substance P content of dorsal root ganglia was assessed as described above.

RESULTS

Effect of Capsaicin on Peripheral Sensory Neuron Function

Sensory Function

Measurement of peripheral sensory function in Hartley guinea pigs 3 days after treatment with a single dose of capsaicin revealed that capsaicin induced marked changes in the ability of the animals to sense nociceptive and non-nociceptive heat and nociceptive chemical stimulation (Table 3). Capsaicin treatment did not result in detectable alterations in the ability of the animals to sense nociceptive or non-nociceptive mechanical, pressure or cold stimulation (Table 3).

Substance P

Substance P content of dorsal root ganglia and dorsal spinal cord in Hartley guinea pigs treated with a single dose of capsaicin (50 mg/kg s.c.) were depleted by 84.2 and 25.4 percent, respectively (Table 4). No change in substance P content of hypothalamus, corpus striatum or ventral spinal cord was observed after capsaicin treatment (Table 4).

Spinal Cord Amino Acid Content

No significant alterations in spinal cord amino acid content were detected in guinea pigs 4 days after treatment with a single dose of capsaicin (50 mg/kg s.c.) (Table 5).

Table 3. Peripheral sensory function in Hartley guinea pigs 3 days after capsaicin treatment (50 mg/kg s.c.)

Test	Function	Mean ± S.E.M. or Percent Responding Vehicle	Capsaicin	P
Hot plate	Nociceptive heat	9.6±1.2	all >30 sec[a]	< 0.001
Skin flinch	Nociceptive heat	13.4±3.5	all >30 sec[a]	< 0.001
Heat probe	Non-nociceptive heat	100%	0%	< 0.001
Zingerone	Nociceptive chemical	100%	0%	< 0.001
Anesthesiometer	Mechanical Distortion	4.2±0.2	4.6±0.2	NS
Air puff	Mechanical Distortion	100%	100%	NS
Paw Press	Nociceptive pressure	0.9±0.1	0.8±0.2 ml	NS
Cold probe	Non-nociceptive cold	100%	100%	NS
Cold plate	Nociceptive	6.9±2.3	4.6±1.2 sec	NS

[a]30 second cutoff to prevent tissue damage

NS - $p < 0.1$ vs. vehicle-treated

Table 4. Substance P immunoreativity (pmoles/g wet weight) in selected neural tissues of Hartley guinea pigs (300-400 g) measured 4 days after capsaicin treatment (50 mg/kg s.c.)

Tissue	Vehicle[a]	Capsaicin	percent change	P
Hypothalamus	121.7 ± 7.9	120.4 ±11.2	-	NS
Corpus striatum	47.4 ± 4.9	36.9 ± 3.7	-	NS
Dorsal root ganglia	16.5 ± 2.2	2.6 ± 0.5	-84.2	.01
Dorsal spinal cord	193.1 ± 8.0	144.8 ± 7.2	-25.4	.01
Ventral spinal cord	28.4 ± 3.9	35.5 ± 2.6	-	NS

[a]vehicle is 10 percent ethanol: 10 percent Tween 80: 80 percent saline.
n= 6 animals/group
Values are mean ± S.E.M.
NS- $p < 0.1$ vs. vehicle-treated

Table 5. Amino acid content of guinea pig dorsal spinal cord (umoles/g wet weight) 4 days after treatment with a single dose of capsaicin (50 mg/kg s.c.).

Amino Acid	Vehicle	Capsaicin	P
Phosphoserine	0.14 ±0.01	0.14 ±0.01	NS
Taurine	2.37 ±0.31	2.32 ±0.17	NS
Phosphoethanolamine	1.27 ±0.14	1.61 ±0.25	NS
Aspartate	1.93 ±0.14	2.07 ±0.15	NS
Threonine	0.13 ±0.03	0.12 ±0.01	NS
Serine	0.54 ±0.08	0.45 ±0.02	NS
Glutamine	1.17 ±0.10	1.49 ±0.10	NS
Glutamate	3.70 ±0.14	3.90 ±0.10	NS
Glycine	3.33 ±0.31	3.06 ±0.10	NS
Alanine	0.52 ±0.03	0.53 ±0.04	NS
GABA	0.78 ±0.10	0.78 ±0.05	NS

n= 4 animals/group

Values are mean ± S.E.M.

NS - $p > 0.1$ vs vehicle-treated

Temporal Relationship Between Substance P Depletion and Antinociception

The temporal relationship between depletion of substance P and the induction of antinociception by capsaicin was investigated by determining the escape latency for guinea pigs from a 55°C hot plate immediately prior to, one day after, and four days after a single injection of capsaicin (50 mg/kg s.c.). Immediately after sensory testing, animals were killed and substance P content of cervical dorsal root ganglia was determined.

Increased escape latency from the 55°C hot plate was observed within 1 day after treatment with capsaicin and lasted throughout the duration of the 4 day experiment (Fig. 8). Alterations in dorsal root ganglia substance P content were not detected 1 day after capsaicin treatment although capsaicin-induced thermal antinociception was present. Substance P content of dorsal root ganglia was found to be significantly decreased 4 days after capsaicin treatment. Thus, depletion of substance P from the central portions of primary afferent neurons is not required for the initiation of thermal antinociception by capsaicin.

Dose-response for Depletion of Substance P and Antinociception

The dose-response relationship for depletion of substance P from primary afferent neurons by capsaicin was investigated in Hartley guinea pigs (350-450 g). Guinea pigs were treated with capsaicin (2-50 mg/kg s.c.) and dorsal root ganglia substance P content was determined 1 day and 4 days after treatment. In addition, the ability to sense

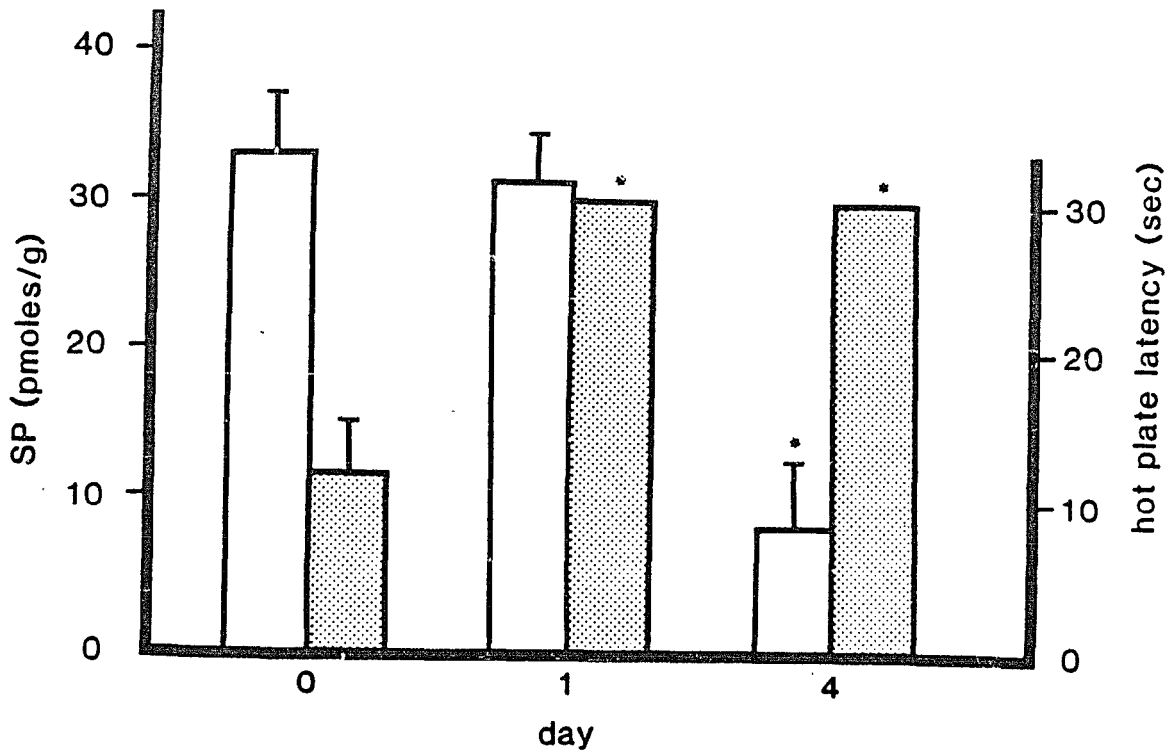


Figure 8. Temporal relationship between the development of substance P depletion and thermal antinociception following a single dose of capsaicin (50 mg/kg s.c.).-- Substance P content of dorsal root ganglia (open bars) and hot plate latency (stipled bars) were measured prior to, 1 day after, and 4 days after capsaicin treatment. Animals were removed from the hot plate after 30 seconds to prevent tissue damage. Values are mean $\pm$ S.E.M. Escape latency after capsaicin treatment shows no variance because all animals reached the predetermined 30 second cutoff time. N=6 animals/group. *$p < 0.05$ vs time zero value by ANOVA and Scheffe's test.

noxious chemical stimuli was assessed by determining the duration of response when 50 ul of a 1 percent (w/v) solution of the chemical irritant zingerone was applied topically to the cornea. Control animals responded by vigorously wiping and scratching the eye for approximately 15 seconds.

Capsaicin did not alter substance P content of dorsal root ganglia 1 day after treatment. However, a dose-dependent decrease in the concentration of substance P in dorsal root ganglia was observed 4 days after capsaicin treatment (Fig. 9). In addition, capsaicin administration produced a dose-dependent decrease in the duration of response when 1 percent zingerone (w/v) was applied to the cornea. This chemogenic antinociception occurred within 1 day of capsaicin administration and was still present to the same degree 4 days after capsaicin treatment (Fig. 9). The ED_{50} dose of capsaicin for the production of antinociception was significantly lower than the dose required for depletion of substance P (Fig. 9).

Effect of Capsaicin on Substance P Synthesis

The effect of capsaicin treatment on the synthesis of substance P was investigated in guinea pigs which were treated with a single dose of capsaicin (50 mg/kg s.c.). Three and one half days after capsaicin treatment the animals received a single injection of ^{3}H-proline. Incorporation of radioactivity into substance P was subsequently measured after isolating substance P by immunoprecipitation and high pressure liquid chromatography (HPLC). Figure 10 shows a typical HPLC

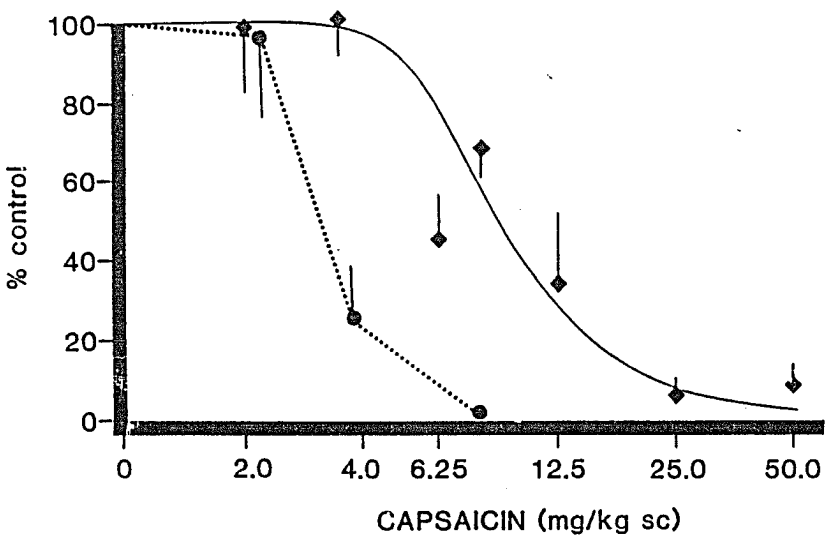


Figure 9. Dose-response relationship for depletion of substance P (———) from dorsal root ganglia when measured 4 days after treatment with capsaicin.-- Also shown is the dose-response relationship for the induction of chemogenic antinociception in the same animals measured by determining the duration of response to topical application of 50 ul of 1 percent zingerone (w/v) to the cornea (----). N=5 animals/point. The curves are statistically different ($p < 0.001$ by 2-way ANOVA). Values are mean $\pm$ S.E.M.

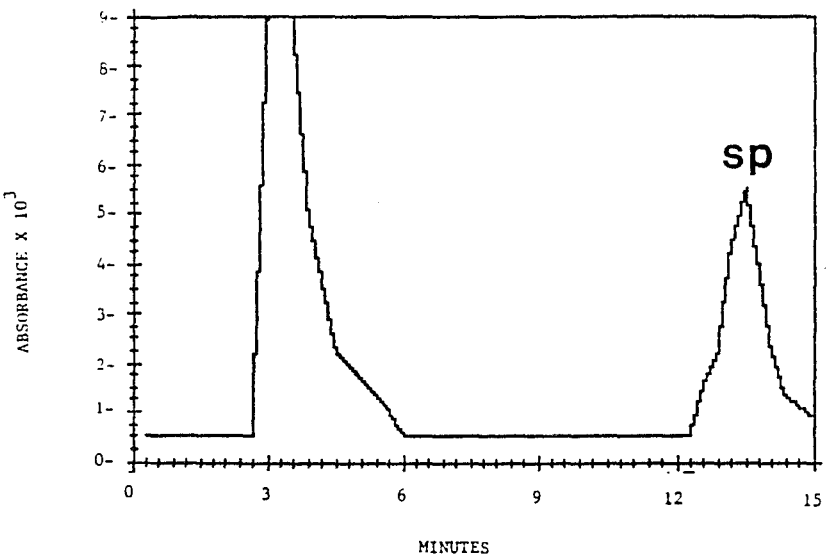


Figure 10. Typical HPLC chromatogram for substance P.-- Mobile phase: 0.1 M potassium phosphate, pH 2.2, plus 25 percent acetonitrile, flow rate: 1.0 ml/min, column: P/N 27324 S/N, detection: 214 nm.

chromatogram for synthetic substance P standard. The long retention time for substance P on the chromatographic system allowed substance P to be separated from all other detectable proteins in guinea pig dorsal root ganglia preparations (Fig. 11). Determining the quantity of radioactivity associated with the substance P peak allowed for the measurement of the rate of substance P synthesis. A typical radioactivity chromatogram is shown in figure 12.

The rate at which substance P was synthesized in dorsal root ganglia of capsaicin and vehicle-treated guinea pigs was found to be linear with time out to at least 24 hours after ^{3}H-proline injection. Capsaicin treatment markedly slowed the rate at which radioactivity was incorporated into substance P when compared to vehicle-treated animals (Fig. 13). Capsaicin-treated animals incorporated radioactivity into substance P in dorsal root ganglia at a rate of 0.48 $\pm$ 0.01 fmoles/day/g dorsal root ganglia (wet weight) as compared to a value of 1.0 $\pm$ 0.02 fmole/day/g in vehicle-treated animals. This represents a 48 percent inhibition in the rate of substance P synthesis which was induced by capsaicin ($p < 0.001$ by 2-way ANOVA).

Effect of Capsaicin on Total Protein Synthesis

The effect of capsaicin treatment on the rate of total protein synthesis in dorsal root ganglia of capsaicin-treated animals was investigated. Guinea pigs were treated with a single dose of capsaicin (50 mg/kg s.c.). Three and one half days after capsaicin treatment the animals received a single injection of ^{3}H-tryptophan. Incorporation of

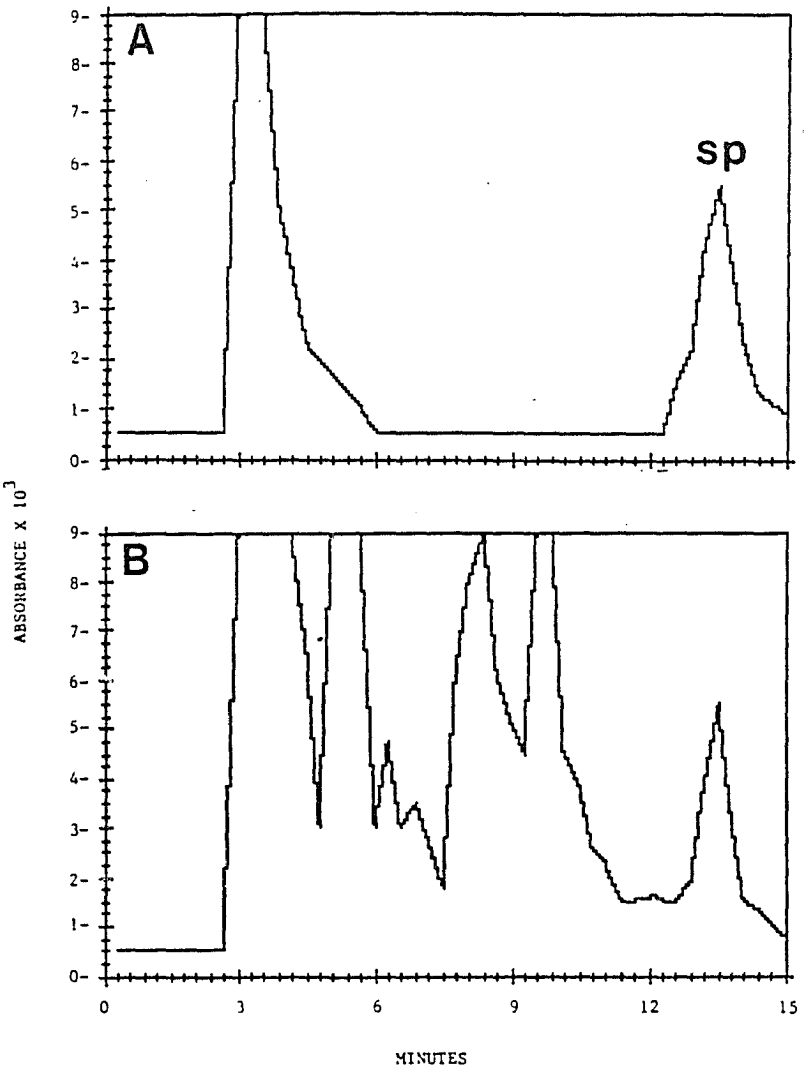


Figure 11. Typical HPLC chromatogram of substance P (SP) standard (A) and chromatogram of substance P from guinea pig dorsal root ganglia following immunoprecipitation (B).-- Mobile phase: 0.1 M potassium phosphate, pH 2.2, plus 25 percent acetonitrile, flow rate: 1.0 ml/min, column: P/N 27324 S/N, detection: 214 nm.

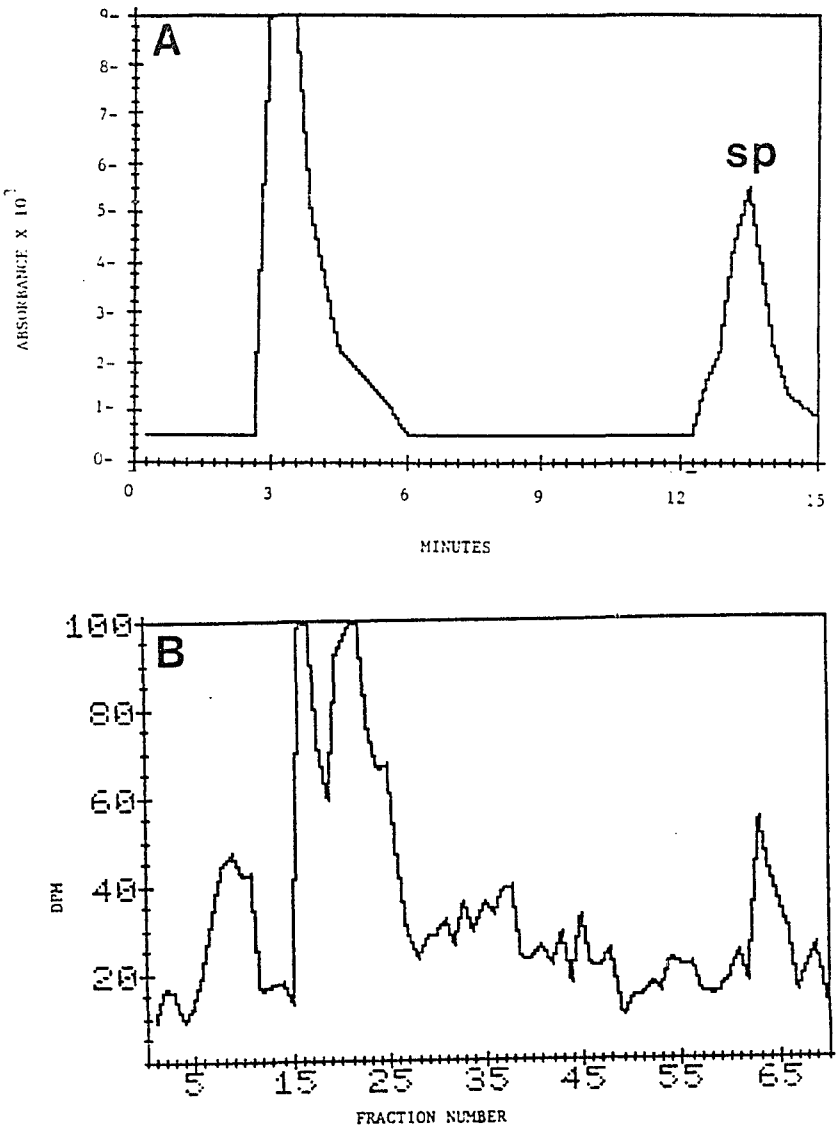


Figure 12. Typical HPLC chromatogram of substance P (SP) standard (A) and chromatogram of ^{3}H-proline incorporated into substance P from guinea pig dorsal root ganglia following immunoprecipitation.-- Mobile phase: 0.1 M potassium phosphate, pH 2.2, plus 25 percent acetonitrile, flow rate: 1.0 ml/min, column: P/N 27324 S/N, detection: A-214 nm, B-liquid scintillation counting.

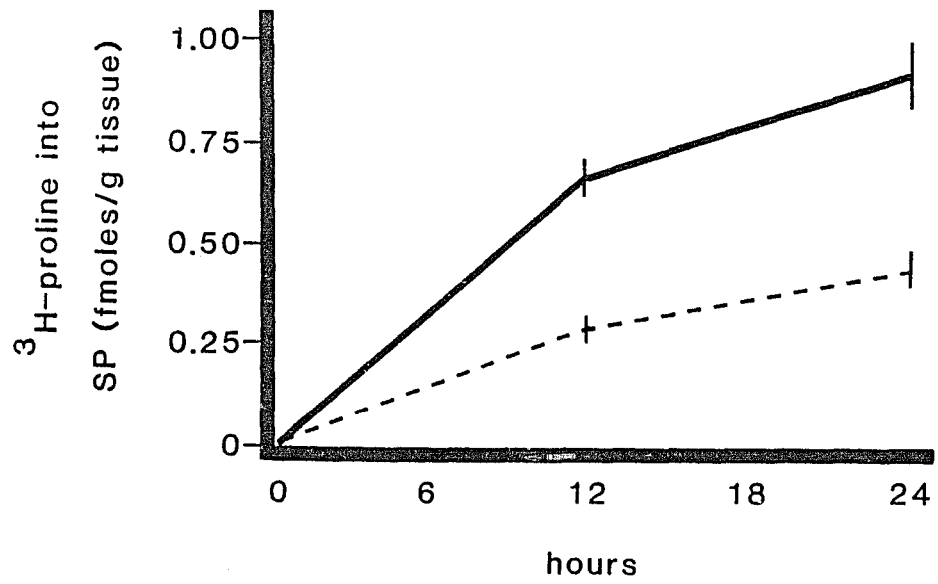


Figure 13. Incorporation of ^{3}H-proline into substance P (SP) in guinea pig dorsal root ganglia of guinea pigs after treatment with capsaicin (50 mg/kg s.c.) (-----) or vehicle (———).-- Values are mean ± S.E.M. N=6 animals/time point.

radioactivity into total protein was measured after exhaustively extracting radioactivity which was not incorporated into protein.

The rate at which radioactivity was incorporated into protein was linear throughout the experiment (Fig 14). Capsaicin treatment produced no detectable effect on the rate at which ^{3}H-tryptophan was incorporated into protein (Fig. 14).

Structure Activity Relationships for Capsaicinoids

Hartley guinea pigs treated with 6 consecutive daily doses of capsaicin, nonylvanylamide or dihydrocapsaicin demonstrated chemogenic (Table 6) and thermal (heat) antinociception (Fig. 15) when measured three days after the last treatment. Pelargonaldehyde and vanillylamide hydrochloride produced no detectable alterations in peripheral sensory function (Table 6 and Fig. 15). Morphine sulfate (10 mg/kg s.c.) altered responses to all sensory stimuli tested (Table 6).

One day after peripheral sensory testing, animals were killed by decapitation and dorsal root ganglia C_4-T_1, dorsal spinal cord C_4-T_1, ventral spinal cord C_4-T_1, hypothalamus, and corpus striatum were removed for determination of substance P content. Capsaicin, nonylvanylamide or dihydrocapsaicin treatment resulted in depletion of substance P immunoreactivity from both dorsal root ganglia (Fig. 16) and dorsal spinal cord (Fig. 17). No compounds tested significantly altered substance P content in hypothalamus (Fig. 18), corpus striatum (Fig. 19) or ventral spinal cord (Fig. 20) when compared to vehicle-treated controls.

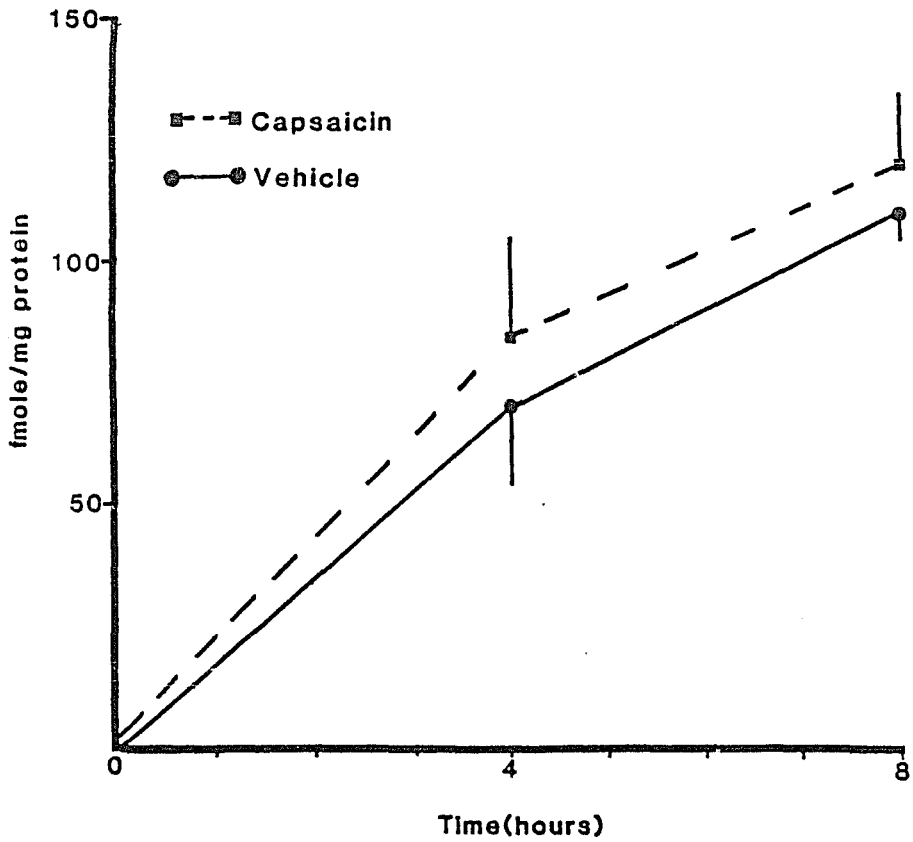


Figure 14. Effect of capsaicin pretreatment on the incorporation of ^{3}H-tryptophan into total protein in guinea pig dorsal root ganglia.

Table 6. Sensory capabilities of guinea pigs after treatment with capsaicin, capsaicin analogs and morphine sulfate.

modality:	Pressure	Chemical	Cold	Heat
stimuli: Treatment	Toe Clamp	Zingerone	Dry-ice	Hot Plate
Vehicle	+	+	+	+
Pelargonaldehyde	+	+	+	+
Vanillylamide HCl	+	+	+	+
Nanoylvanillamide	+	-	+	-
Dihydrocapsaicin	+	-	+	-
Capsaicin	+	-	+	-
Morphine	-	-	-	-

+ = Response to stimuli
- = No response to stimuli

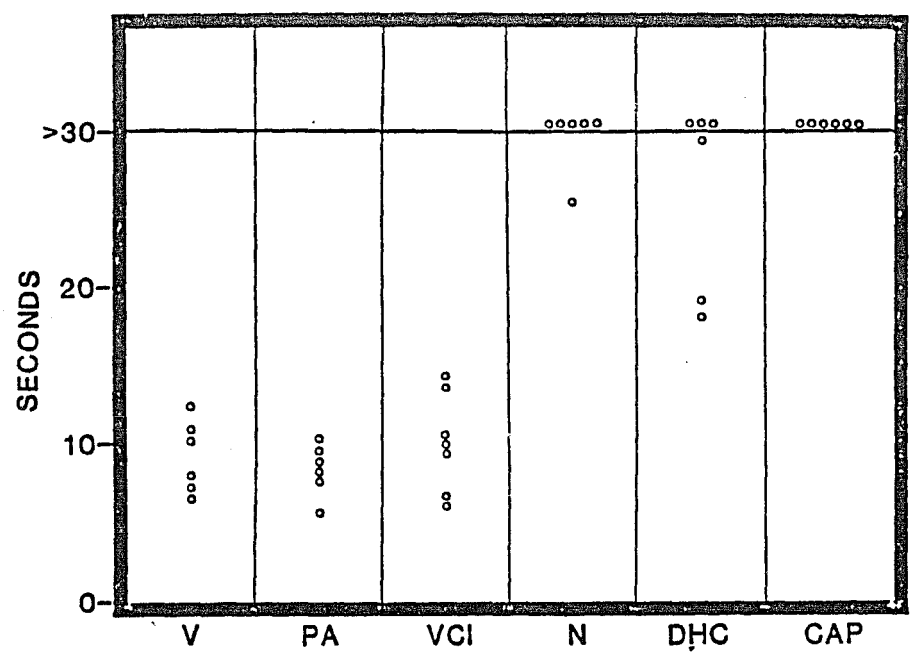


Figure 15. Hot plate test on guinea pigs pretreated with vehicle (V), pelargonaldehyde (PA), vanillylamide hydrochloride (VCl), nanoylvanillamide (N), dihydrocapsaicin (DHC) or capsaicin (CAP).-- A 30 second "cutoff" was used to prevent severely burning the animal's feet. Points represent individual animals.

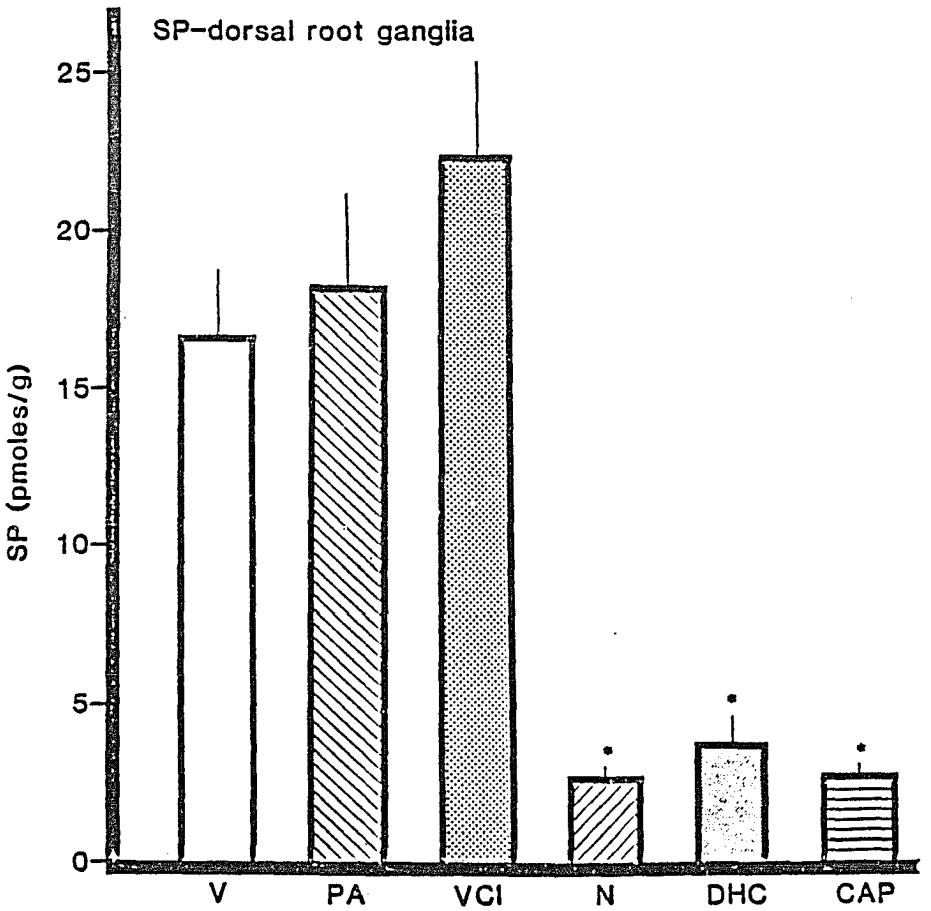


Figure 16. Substance P immunoreactivity (SP) in dorsal root ganglia of guinea pigs pretreated with vehicle (V), pelargonaldehyde (PA), vanillylamide hydrochloride (VCl), nanoylvanillamide (N), dihydrocapsaicin (DHC) or capsaicin (CAP).-- N=6 animals/ group. Values are mean $\pm$ S.E.M. *$p < 0.05$ by ANOVA and Scheffe's test.

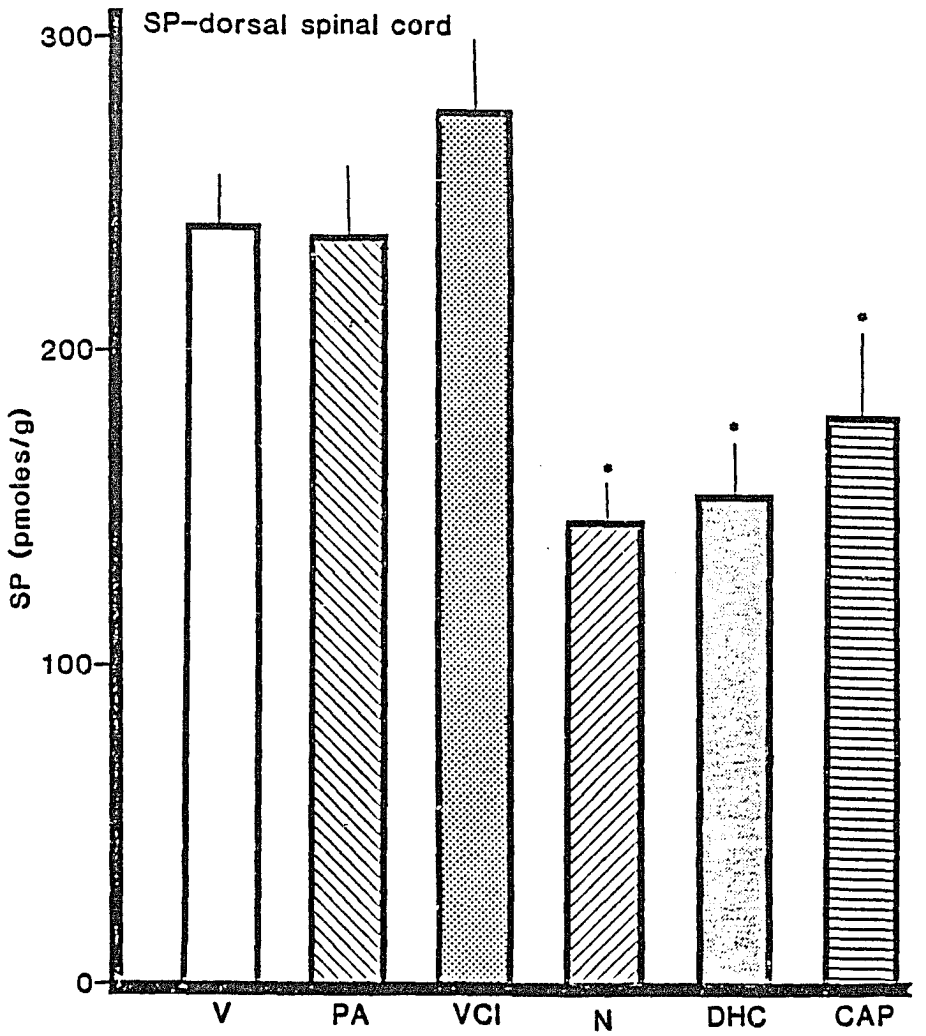


Figure 17. Substance P immunoreactivity (SP) in dorsal spinal cord of guinea pigs pretreated with vehicle (V), pelargonaldehyde (PA), vanillylamide hydrochloride (VCl), nanoylvanillamide (N), dihydrocapsaicin (DHC) or capsaicin (CAP).-- N=6 animals/ group. Values are mean $\pm$ S.E.M. *$p < 0.05$ by ANOVA and Scheffe's test.

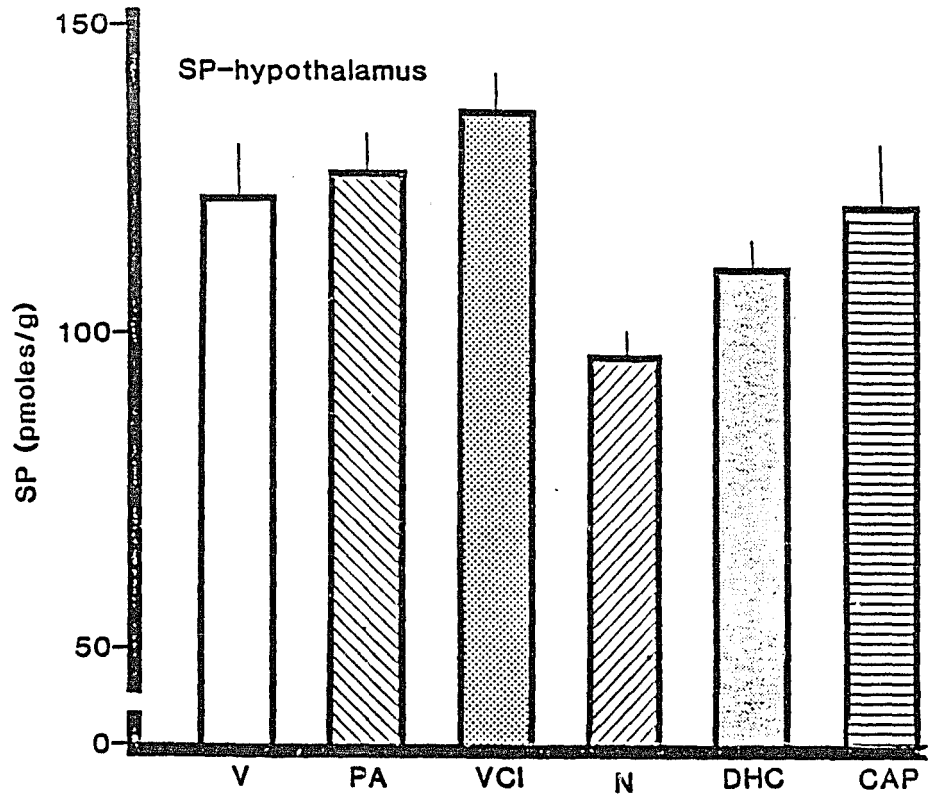


Figure 18. Substance P immunoreactivity (SP) in hypothalamus of guinea pigs pretreated with vehicle (V), pelargonaldehyde (PA), vanillylamide hydrochloride (VCl), nanoylvanillamide (N), dihydrocapsaicin (DHC) or capsaicin (CAP).-- N=6 animals/ group. Values are mean $\pm$ S.E.M.

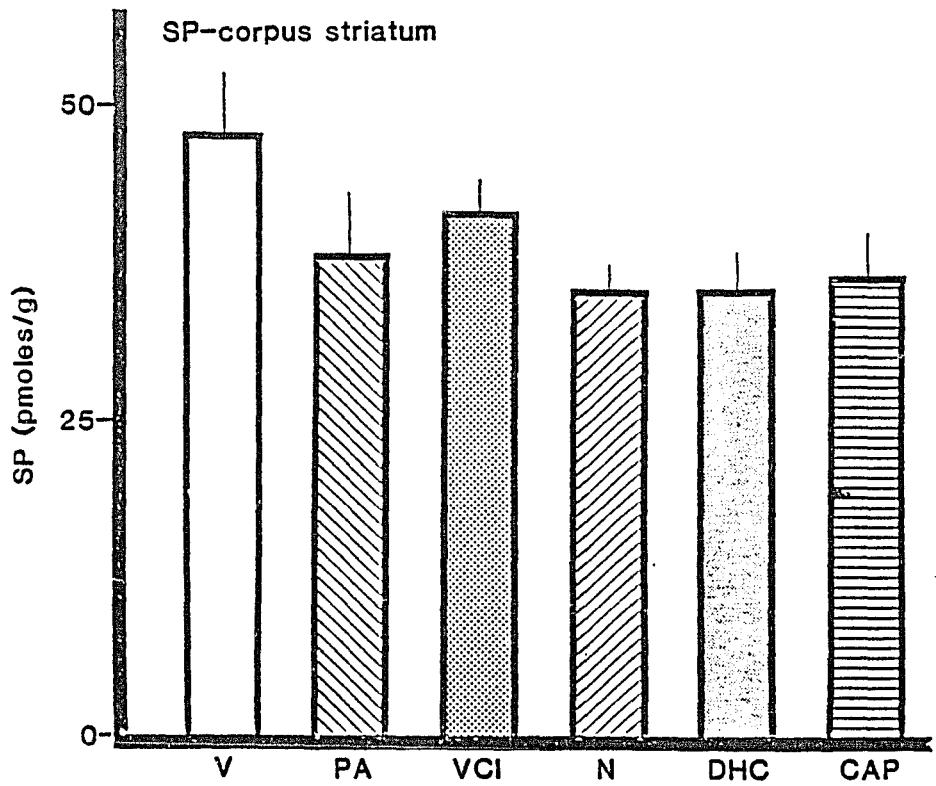


Figure 19. Substance P immunoreactivity (SP) in corpus striatum of guinea pigs pretreated with vehicle (V), pelargonaldehyde (PA), vanillylamide hydrochloride (VCl), nanoylvanillamide (N), dihydrocapsaicin (DHC) or capsaicin (CAP).-- N=6 animals/ group. Values are mean $\pm$ S.E.M.

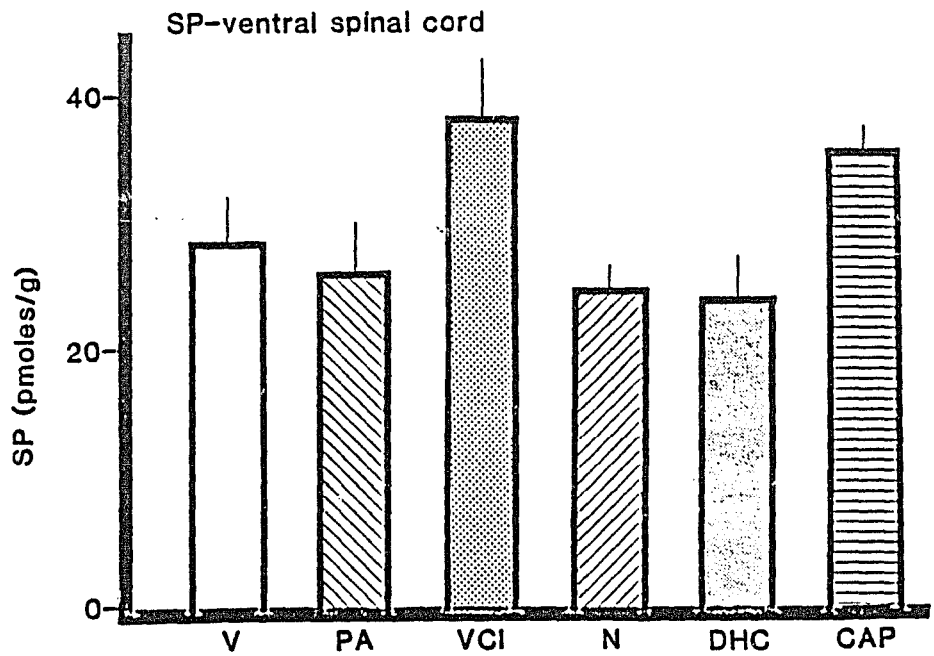


Figure 20. Substance P immunoreactivity (SP) in ventral spinal cord of guinea pigs pretreated with vehicle (V), pelargonaldehyde (PA), vanillylamide hydrochloride (VCl), nanoylvanillamide (N), dihydrocapsaicin (DHC) or capsaicin (CAP).-- N=6 animals/ group. Values are mean $\pm$ S.E.M.

Pharmacology of Dihydrocapsaicin

Effect of Dihydrocapsaicin on Thermoregulation

Parenteral administration of either capsaicin or dihydrocapsaicin to rats resulted in a rapid hypothermia. Hypothermia was maximal approximately 2 hours after treatment and returned to baseline within 6 hours. The dose-response relationship for the production of hypothermia by either capsaicin or dihydrocapsaicin is shown in figure 21. Although dihydrocapsaicin and capsaicin were equipotent in producing hypothermia, dihydrocapsaicin was 65 percent more effective ($p < 0.05$ by ANOVA). In addition, the efficacy with which both capsaicin and dihydrocapsaicin produced hypothermia decreased with repeated administration (Fig. 22). After repeated high dose administration, total desensitization to the hypothermic effects of both capsaicin and dihydrocapsaicin was observed (Fig. 22). In addition, no hypothermic response was detected when capsaicin (2.0 mg/kg s.c.) was administered to rats previously desensitized with dihydrocapsaicin. Similiarly, dihydrocapsaicin (2.0 mg/kg s.c.) produced no response when administered to rats previously desensitized with capsaicin.

Effect of Dihydrocapsaicin on Nociception

Chemogenic nociception, as assessed by application of 1 percent zingerone to the cornea, was determined in rats treated with capsaicin, dihydrocapsaicin or vehicle. Vehicle-treated rats responded to zingerone by intensely wiping the eye for a period of approximately 10 sec. Response to zingerone was completely abolished when measured 1

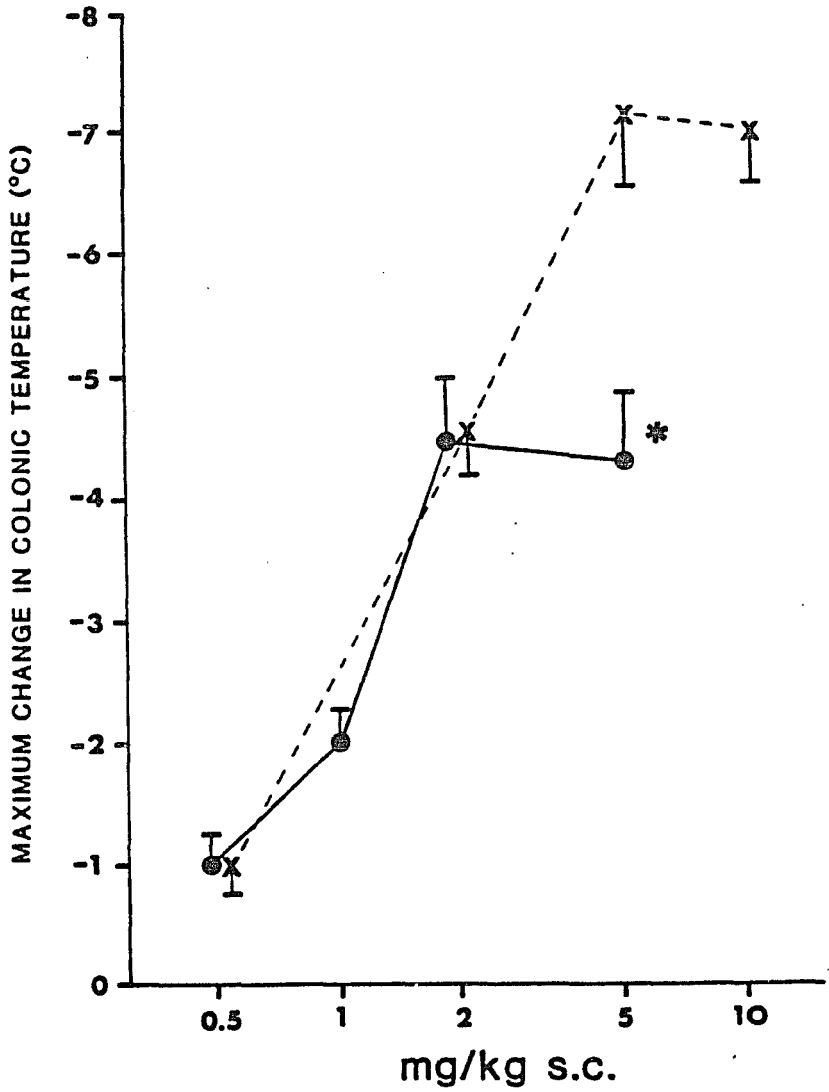


Figure 21. Dose-response relationship for the production of hypothermia by capsaicin (———) and dihydrocapsaicin (-----).-- Vehicle produced no change in colonic temperature. Values are mean ± S.E. N=6 animals per time point. *$p < 0.05$ vs dihydrocapsaicin-treated by ANOVA and Scheffe's test.

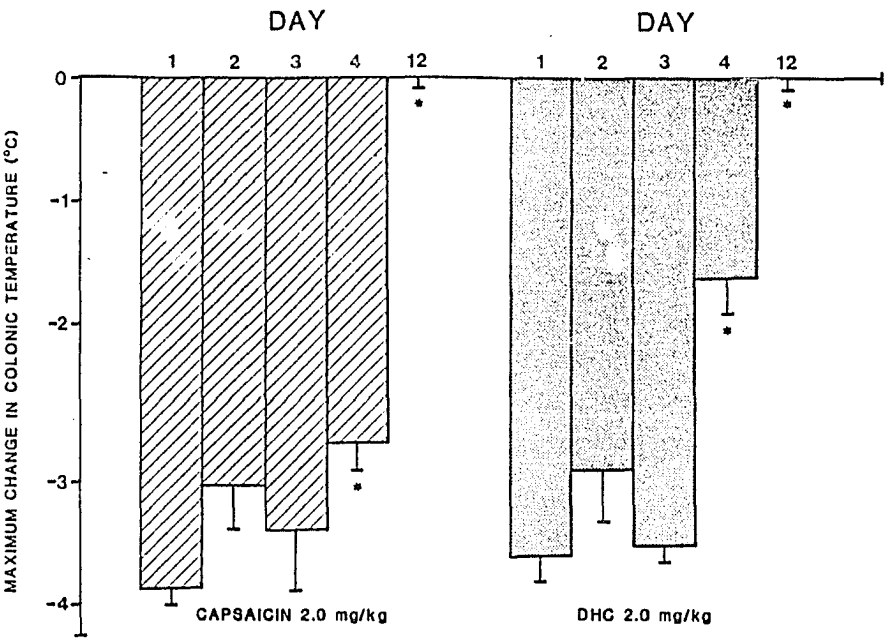


Figure 22. Desensitization to the thermoregulatory effects of capsaicin and dihydrocapsaicin (DHC).-- Treatments are as described in Table 2 (see Methods). Values are mean ± S.E.M. N=6 animals/group. *$p < 0.05$ vs. day 1 value by ANOVA and Scheffe's test.

day or 5 days after treatment with capsaicin or dihydrocapsaicin (10, 50, 100, 100, 100, 100 mg/kg/day) although thermal nociception was not markedly changed (Table 7).

Effect of Dihydrocapsaicin on Tissue Substance P

Measurement of tissue substance P content 1 day after sensory testing revealed that both capsaicin and dihydrocapsaicin administration markedly depleted substance P from dorsal root ganglia and dorsal spinal cord. Substance P content of non-primary afferent tissues was unchanged (Table 8).

Biotransformation of Capsaicin and Dihydrocapsaicin

Capsaicin interacted with rat hepatic microsomes to produce a type I difference spectrum. The extent of the interaction was concentration dependent. Eadie-Hostee analysis revealed a K_s for spectral interaction of capsaicin with hepatic microsomes of approximately 8 uM (Fig. 23).

Although rat hepatic microsomes readily demethylated ethylmorphine, no demethylation of capsaicin was detectable (Fig. 24). Addition of equimolar quantities of capsaicin to ethylmorphine incubations resulted in complete inhibition of ethylmorphine demethylation (Fig. 24). More detailed inhibition studies revealed that capsaicin inhibited ethylmorphine demethylation in a manner which displayed both competitive and non-competitive characteristics (Table 9). The potency with which capsaicin inhibited ethymorphine demethylation was approximately equal to that of SKF 525A (Table 9).

Table 7. Tail flick latencies (mean ± S.E.M.) of Sprague-Dawley rats (200-250 g) 1 day and 5 days after animals were treated with 6 consecutive daily doses of vehicle, capsaicin or dihydrocapsaicin (DHC)(10, 50, 100, 100, 100, 100 mg/kg s.c.).

	Vehicle	Capsaicin	p*	DHC	p*
1 day	5.4 ±0.3	6.2 ±0.3	< 0.03	6.2 ±0.1	< 0.02
5 days	5.2 ±0.2	5.9 ±0.3	< 0.05	6.1 ±0.3	< 0.01

N= 6 animals/group
*vs vehicle treated by ANOVA and Scheffe's test

Table 8. Substance P immunoreactivity in rats following 6 consecutive daily doses of vehicle, capsaicin or dihydrocapsaicin (10, 50, 100, 100, 100, 100 mg/kg s.c.). Values are pmoles substance P/g wet weight ± S.E.

	Vehicle	Capsaicin	p*	dihydrocapsaicin	p*
hypothalamus	152.8 ±18.9	147.3 ±18.4	NS	162.1 ± 9.9	NS
corpus striatum	37.5 ±3.7	44.4 ±9.0	NS	41.0 ±5.5	NS
dorsal root ganglia	5.7 ±0.5	3.3 ±0.7	< 0.02	3.7 ±0.8	< 0.04
dorsal cord	167.6 ±19.4	124.6 ± 8.2	< 0.05	128.6 ± 6.4	< 0.03
ventral cord	12.6 ±1.3	14.8 ±2.2	NS	16.2 ±2.2	NS

N = 6 animals group.
*vs vehicle-treated by ANOVA and Scheffe's test.
NS - $p < 0.05$ vs. vehicle-treated.
Values are mean ± S.E.M.

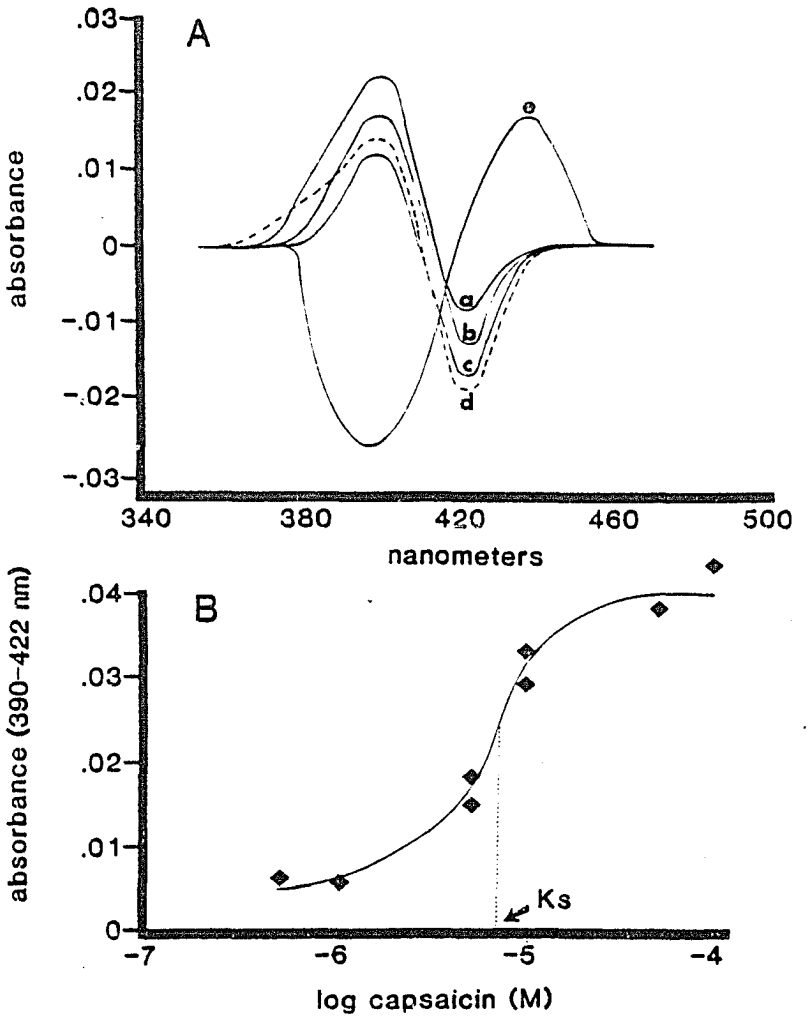


Figure 23. Spectral interaction of capsaicin with rat hepatic microsomes.-- A. Type I difference spectra which result when capsaicin (5×10^{-6} M (a), 5×10^{-5} M (b), 5×10^{-4} M (c)) interacts with hepatic microsomes. Amino pyrene (7 mM)(d) and aniline (7 mM)(e) were included as controls. B. Concentration dependent nature of spectral interaction. The K_s (concentration for one half maximal absorbance) equaled 8 uM.

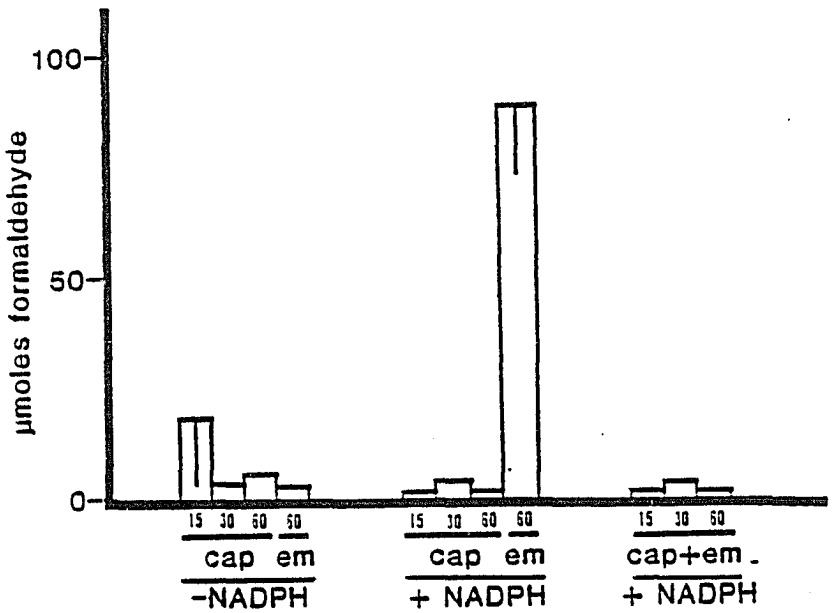


Figure 24. Formaldehyde production by rat liver microsomes incubated with 1 mM capsaicin (CAP) and/or 1 mM ethylmorphine (EM) for 15, 30 and 60 minutes with and without the NADPH generating system.-- No detectable demethylation of capsaicin occurred and the addition of capsaicin completely inhibited demethylation of ethylmorphine. Values are mean $\pm$ S.E.M. N=3 incubations/time point.

Table 9. Effects of capsaicin and SKF 525A on Eadie-Hofstee kinetic constants for the production of formaldehyde resulting from demethylation of ethylmorphine by rat liver microsomes.

INHIBITOR CONCENTRATION (uM)				
CAPSAICIN	SKF 525A	r	Km (mM)	Vmax (nmoles/mg protein)
-	-	0.999	1.83 ±0.05	6.0 ±0.2
25	-	0.999	2.78* ±0.03	5.3* ±0.4
50	-	0.987	3.22* ±0.04	4.6* ±0.2
-	25	0.999	2.85* ±0.07	5.4* ±0.6

*$p < 0.05$ vs. no inhibitor by ANOVA and Scheefe's test.

Values are means of 4 experiments ± S.E.M.

Inhibition of biotransformation *in vivo* by capsaicin was investigated by determining sleeping time in response to sodium pentobarbital (30 mg/kg i.p.). A single treatment with capsaicin (10 mg/kg s.c.) significantly ($p < 0.001$ by Student's t-test) prolonged pentobarbital-induced sleeping time (Fig. 25).

Since radiolabelled capsaicin is not available, covalent binding of capsaicinoids was investigated using ^{3}H-dihydrocapsaicin as substrate for microsomal biotransformation systems. ^{3}H-dihydrocapsaicin interacted in a non-extractable manner with microsomal protein at a rate of approximately 60 pmoles/mg protein/hour (Fig. 26). Heat denaturation, removal of NADPH generating system, addition of reduced glutathione (1 mM) or addition of equimolar SKF 525A (1 mM) significantly inhibited the apparent covalent binding of ^{3}H-dihydrocapsaicin to microsomal protein (Fig. 26).

Analysis of microsomal metabolites of capsaicin and dihydrocapsacin by thin layer chromatography revealed a single peak of radioactivity for each substrate (Fig. 27). The presence of these peaks, which comigrated, was dependent upon the existence of a complete microsomal metabolizing system.

The distribution to selected tissues and *in vivo* covalent binding of ^{3}H-dihydrocapsaicin was investigated in rats following administration of dihydrocapsaicin (10 mg/kg s.c.) containing 5 uCi of ^{3}H-dihydrocapsaicin. The maximum concentration of ^{3}H-dihydrocapsaicin in the liver occurred 6 hours after injection. Brain and spinal cord concentrations did not approach maximum values until 24 hours after

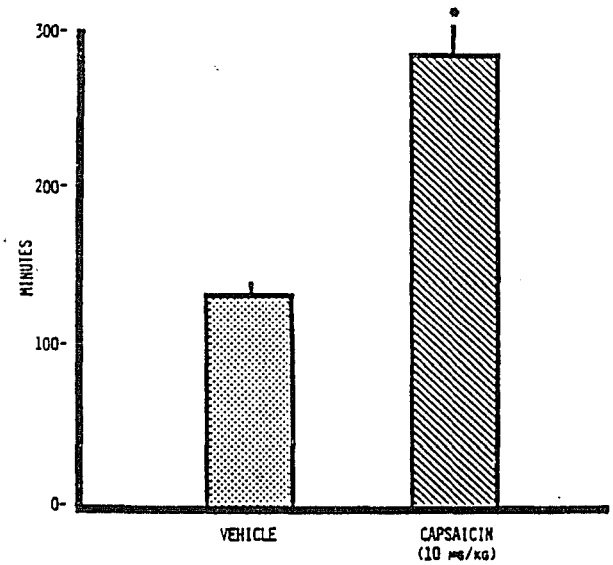


Figure 25. Effect of capsaicin (10 mg/kg s.c.) on sodium pentobarbital (30 mg/kg i.p.) sleep time.-- Capsaicin was administered 6 hours before pentobarbital. N=5 animals/group. Values are mean ± S.E.M. *$p < 0.05$ vs vehicle-treated by Student's t-test.

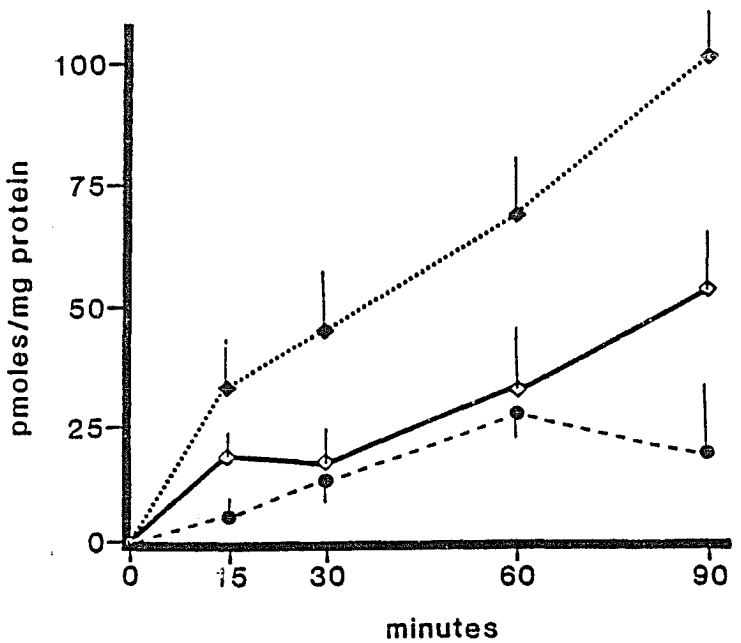


Figure 26. Covalent binding of ^{3}H-dihydrocapsaicin to microsomal protein during in vitro incubation.-- Radioactivity bound to hepatic microsomes in an unextractable manner at a rate of approximately 60 pmoles/ mg protein/hour (·····). Binding was inhibited by omission of the NADPH generating system or by prior heat denaturation on microsomal protein. Addition of reduced glutathione (1 mM)(------) of SKF 525A (1 mM) (———) markedly inhibited binding. Points are mean ± S.E.M. after subtraction of value for heat denatured microsomes. N=3 incubations/ time point.

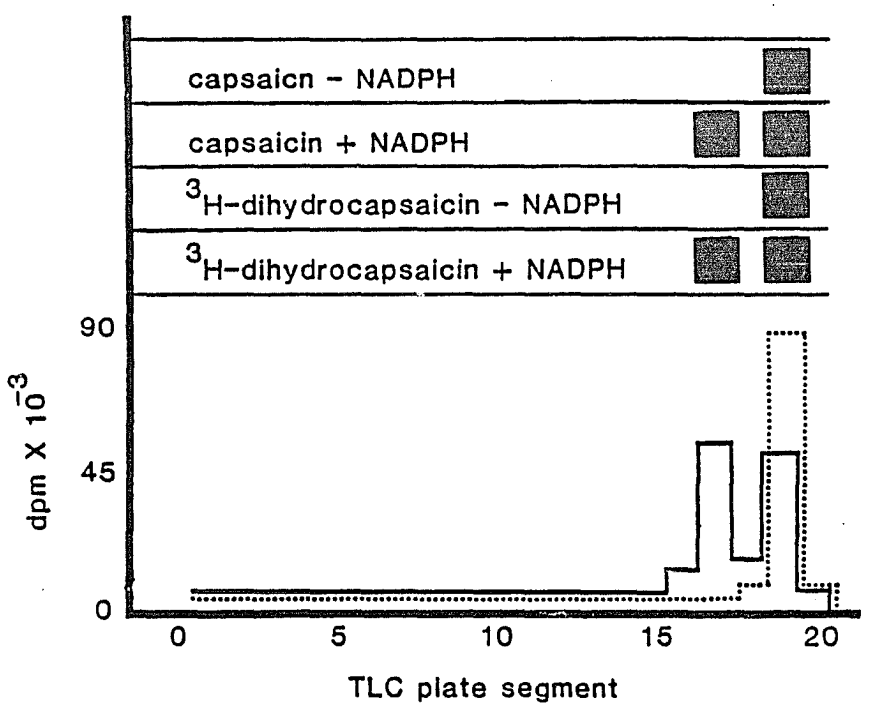


Figure 27. Thin layer chromatography of capsaicin, ^{3}H-dihydrocapsaicin and metabolites.-- Microsomal incubations of capsaicin (1 mM) or ^{3}H-dihydrocapsaicin (1 mM) with and without NADPH generating system were chromatographed on LKD (80 A) silica gel plates. Mobile phase was ethylacetate: acetic acid (9:1). Upper frames show spots detected after plates were treated with 1 percent 2,6-dichloroquinone-4-chloroimide and ammonia. Lower frame shows radioactivity profile following chromatography of microsomal incubations of ^{3}H-dihydrocapsaicin with (———) or without (------) NADPH generating system.

treatment (Fig. 28). ^{3}H-dihydrocapsaicin covalently bound (ie. non-extractable) to hepatic protein attaining a maximum value of approximately 55 pmoles/mg protein 12 hours after treatment. Binding to total hepatic protein was equal to that measured in the S-9 fraction (Fig. 29). No non-extractable radioactivity was detected in brain or spinal cord homogenates.

Site of Action for Dihydrocapsaicin-induced Antinociception

Effect of Local Administration of Dihydrocapsaicin

Guinea pigs treated with 8 ug of ^{3}H-dihydrocapsaicin (18 uCi) by unilateral injection into a front footpad demonstrated localized thermal antinociception surrounding the site of injection as measured by failure to withdraw from the hot probe (Table 10). This localized antinociception occurred within 2 hours of ^{3}H-dihydrocapsaicin injection and lasted at least 10 days. Substance P content (Fig. 30) and levels of ^{3}H-dihydrocapsaicin equivalents (Fig. 31) in ipsilateral dorsal root ganglia C_4-T_1 were not statistically different from levels in contralateral dorsal root ganglia at any time measured (2 hours - 10 days). Levels of total ^{3}H-dihydrocapsaicin equivalents (Fig. 32) in skin obtained from feet treated with ^{3}H-dihydrocapsaicin were initially greater, but within 24 hours fell to values which were not statistically different from those in skin of contralateral vehicle injected feet. In contrast, levels of unextractable radioactivity in skin from feet treated with ^{3}H-dihydrocapsaicin were consistently 3-4 fold higher than levels in skin from contralateral vehicle injected feet (Fig. 33).

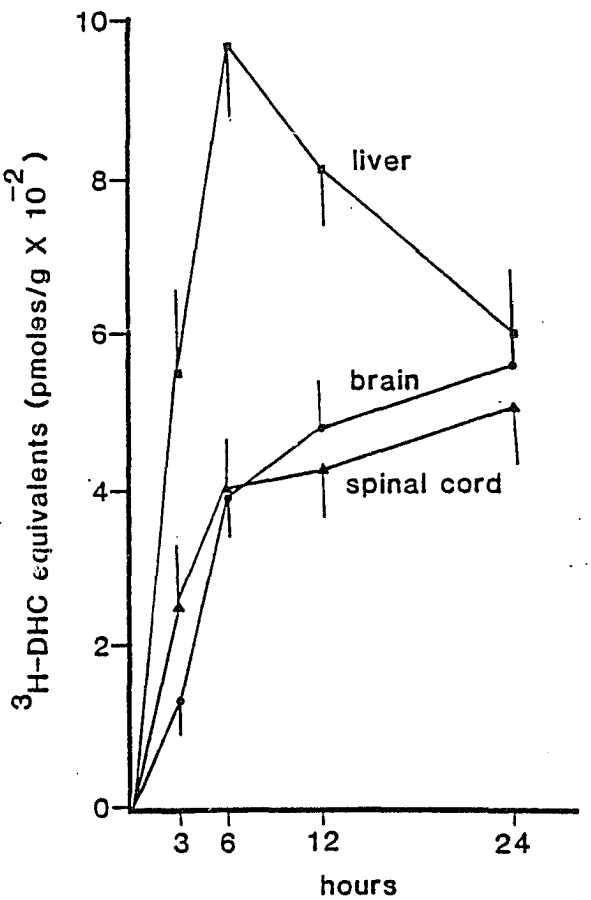


Figure 28. Distribution of radioactivity to brain, spinal cord and liver of rats treated with ^{3}H-dihydrocapsaicin (10 mg/kg s.c.; 5 uCi).-- N= 5 animals/ time point. Values are mean ± S.E.M.

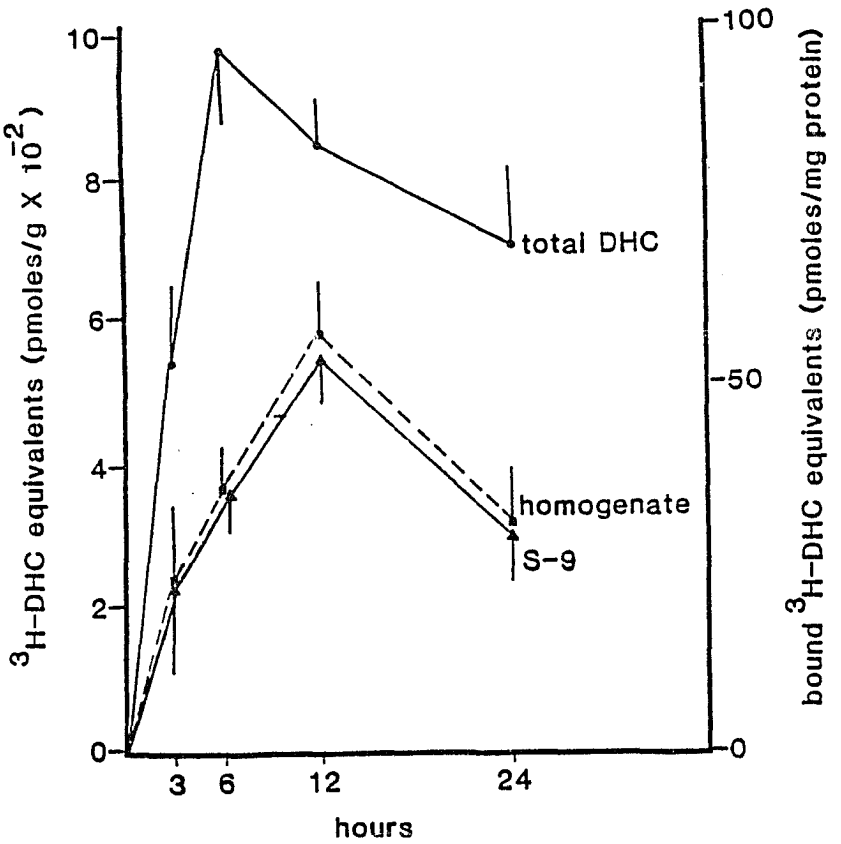


Figure 29. Covalent binding of ^{3}H-dihydrocapsaicin to rat liver protein.-- Animals received ^{3}H-dihydrocapsaicin (10 mg/kg s.c.; 5 uCi). Unextractable radioactivity was determined in protein from whole liver homogenates (■,-----) and S-9 fractions (▲,———) at various times after dihydrocapsaicin treatment. Levels of total radioactivity are also shown (●,———). All values are mean ± S.E.M. N=5 animals/ group.

Table 10. Effect of local administration of ^{3}H-dihydrocapsaicin on response to hot probe.- A front footpad of guinea pigs was injected with 8 ug of ^{3}H-dihydrocapsaicin. The contralateral foot was injected with vehicle. The response of each foot to a hot probe was assessed at various times after treatment.

	RESPONSE TO HOT PROBE					
	HOURS			DAYS		
	2	8	24	4	6	10
^{3}H-dihydrocapsaicin-treated	-	-	-	-	-	-
Vehicle-treated	+	+	+	+	+	+

- = No response
+ = Response within 3 seconds

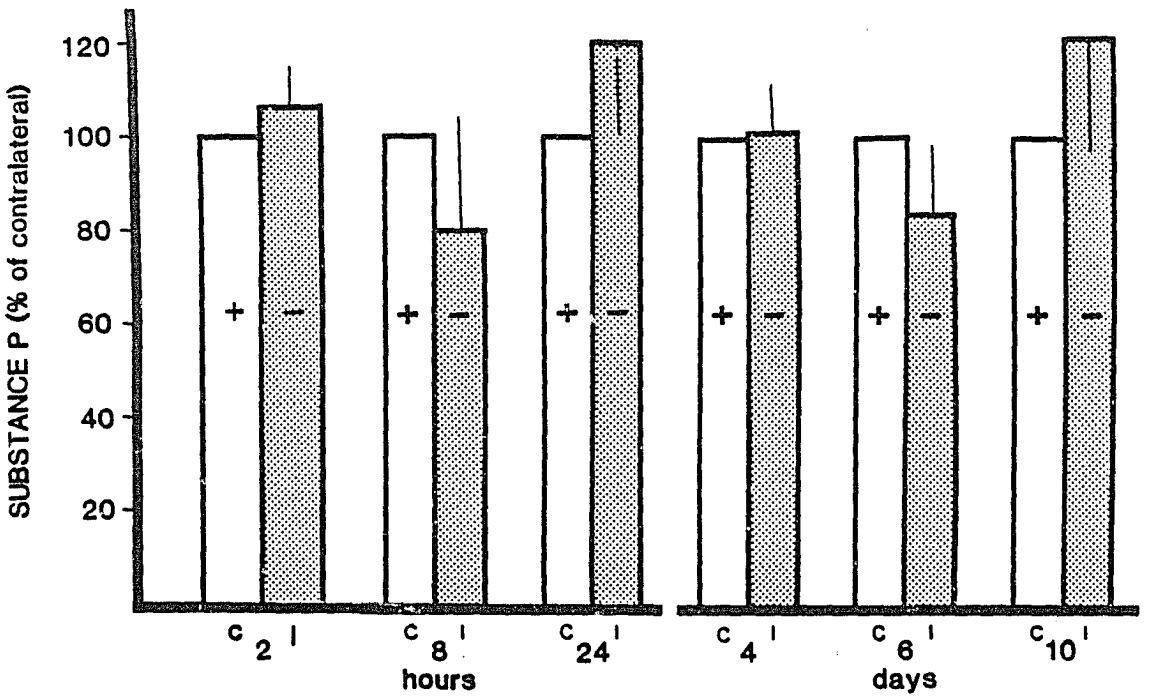


Figure 30. Substance P content of ipsilateral (I) and contralateral (C) dorsal root ganglia C_4-T_1 at various times after injection of 8 ug of ^{3}H-dihydrocapsaicin into a front footpad.-- N= 5 animals/ time point.

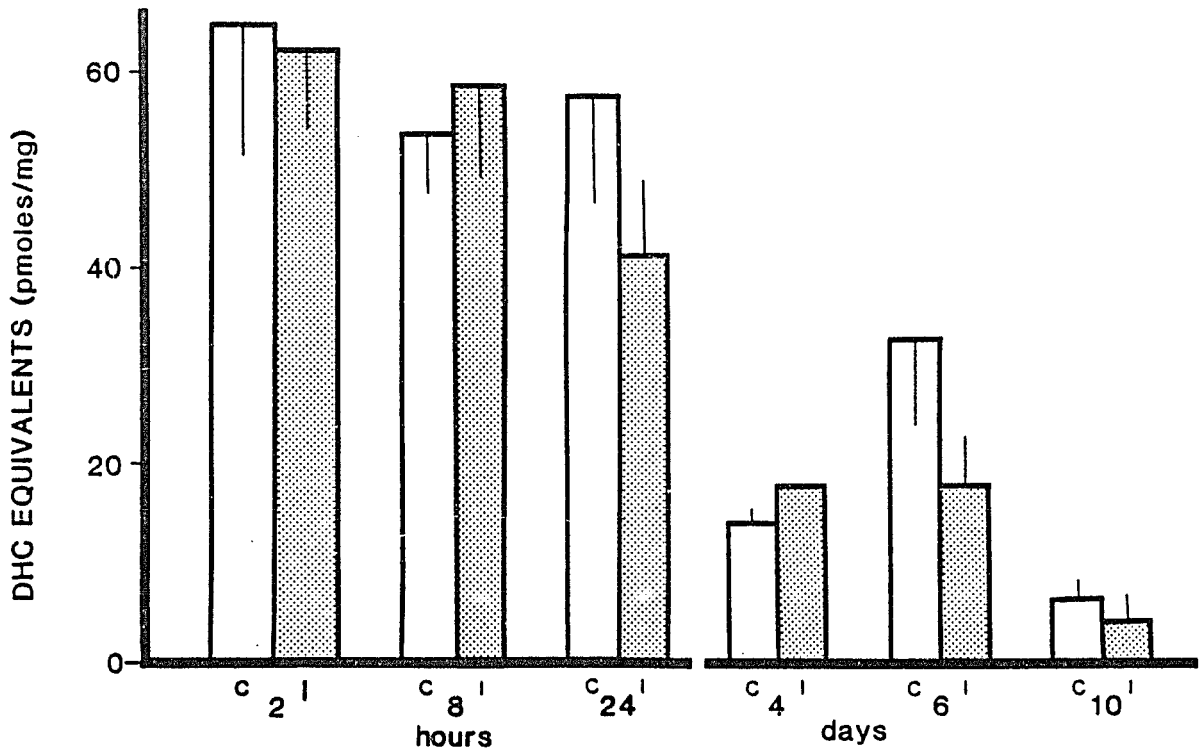


Figure 31. ^{3}H-dihydrocapsaicin equivalents (mean + S.E.M.) in ipsilateral (I) and contralateral (C) dorsal root ganglia C_4-T_1 at various times after injection of 8 ug of ^{3}H-dihydrocapsaicin into a front footpad.-- N= 5 animals/ time point.

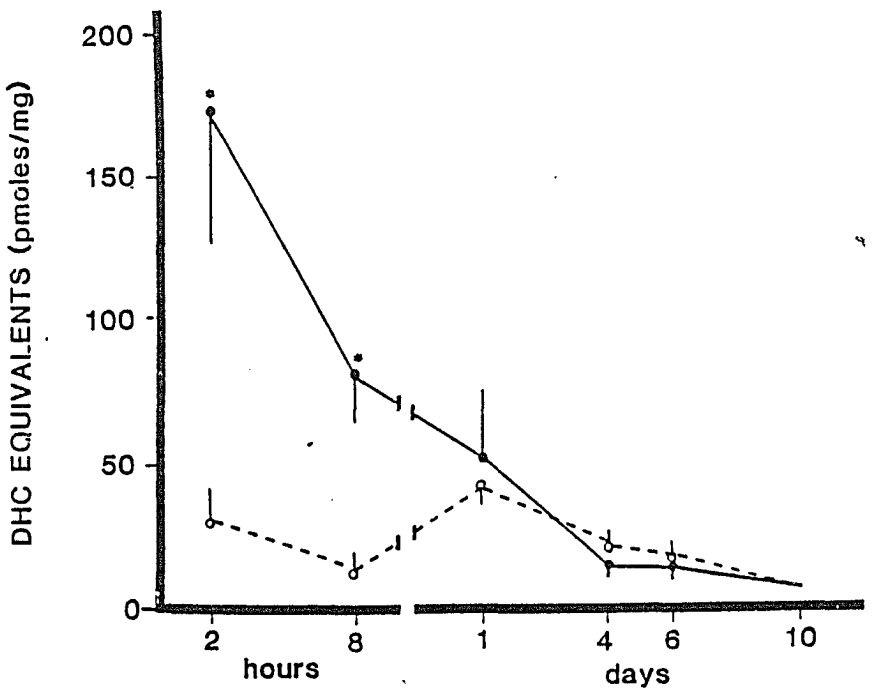


Figure 32. ^{3}H-dihydrocapsaicin equivalents (mean + S.E.M.) in ipsilateral (------) and contralateral (———) front footpad skin at various times after injection of 8 ug of ^{3}H-dihydrocapsaicin into a front footpad.-- N= 5 animals/ time point. *p < 0.05 vs. contralateral value by ANOVA.

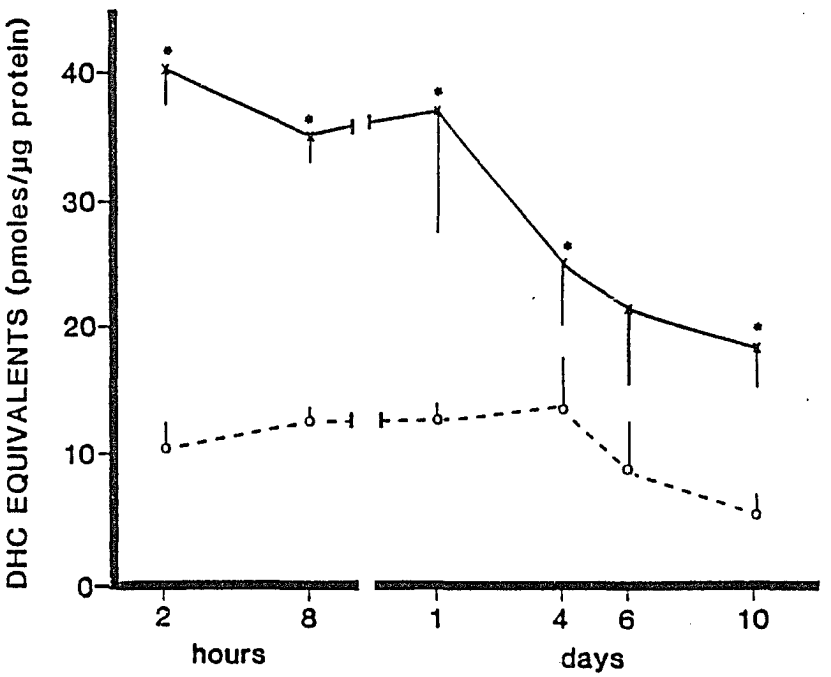


Figure 33. Unextractable ^{3}H-dihydrocapsaicin equivalents (mean ± S.E.M.) in ipsilateral (------) and contralateral (————) front footpad skin at various times after injection of 8 ug of ^{3}H-dihydrocapsaicin into a front footpad.-- N= 5 animals/ time point. *$p < 0.05$ vs. contralateral value by ANOVA.

Mechanism of Substance P Depletion

Effect of Systemic Capsaicin on the Retrograde Transport of NGF

To investigate the effect of capsaicin treatment on the retrograde axoplasmic transport of NGF, guinea pigs received a single unilateral injection of ^{125}I-NGF (1 uCi) in a front footpad. Transport of NGF was then determined at various times after peripheral NGF injection by assessing the quantity of ^{125}I in ipsilateral dorsal root ganglia C_4-T_1 minus the quantity of ^{125}I in contralateral dorsal root ganglia. ^{125}I-NGF appeared in ipsilateral dorsal root ganglia within 4 hours of peripheral injection. Maximum concentration of ^{125}I-NGF in ipsilateral dorsal root ganglia was observed 24 hours after peripheral injection (Fig 34).

The effects of capsaicin on the time course for the retrograde axoplasmic transport of NGF was investigated in guinea pigs 4 days after treatment with a dose of capsaicin (50 mg/kg s.c.) which would maximally deplete substance P from primary afferent neurons. As with controls, ^{125}I-NGF was detected in dorsal root ganglia of capsaicin-treated animals within 4 hours and maximum concentrations were attained 24 hours after peripheral injection (Fig. 34). In contrast to controls, the maximum concentration of ^{125}I-NGF in dorsal root ganglia of capsaicin-treated animals was approximately 45 percent of that observed in vehicle-injected control animals ($p < 0.05$ by ANOVA).

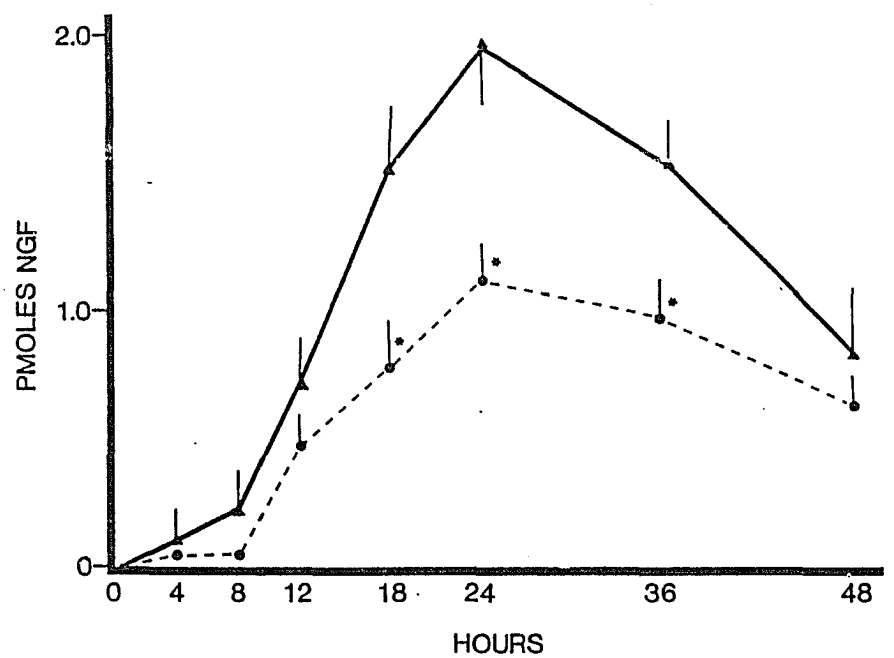


Figure 34. Time-course for the accumulation of radioiodine in ipsilateral dorsal root ganglia C_4-T_1 4 days after treatment with capsaicin (------) or vehicle (———).-- Values are mean ± S.E.M. *$p < 0.05$ vs vehicle-treated by ANOVA.

Dose-response for Inhibition of NGF Transport

As 24 hours after peripheral injection was the time at which maximal concentrations of ^{125}I-NGF were reached in dorsal root ganglia of both control and capsaicin-treated animals, the 24 hour time point was utilized for dose-response studies. A dose-dependent inhibition of the retrograde axoplasmic transport of ^{125}I-NGF was observed both 1 day and 4 days after capsaicin treatment. Doses of capsaicin that depleted substance P from dorsal root ganglia (Fig. 9) also inhibited the retrograde transport of NGF (Fig. 35). Further, inhibition of the retrograde axoplasmic transport of NGF was present 1 day after capsaicin treatment (Fig. 35) although substance P was not depleted. This indicates that alterations in the retrograde axoplasmic transport of NGF preceded the depletion of substance P.

Effect of Locally Administered Capsaicin on NGF Transport

Administration of capsaicin into a footpad 4 days prior to injection of ^{125}I-NGF at the same site had no measurable effect on the accumulation of ^{125}I-NGF in ipsilateral dorsal root ganglia (Fig. 36).

Effect of NGF Supplementation

The cause and effect relationship between depletion of substance P and alterations in the retrograde axoplasmic transport of NGF was investigated in guinea pigs which were treated with capsaicin and supplemented with mouse NGF. Administration of NGF completely blocked capsaicin-induced depletion of substance P (Fig. 37) and, in fact, may have increased substance P levels above control in the

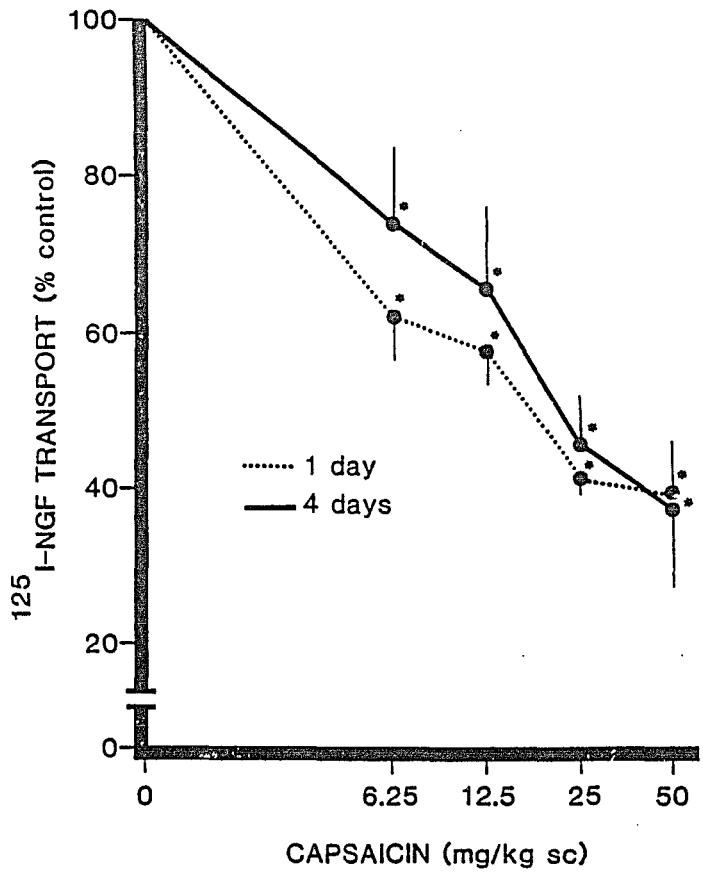


Figure 35. Dose-response relationship for inhibition of the retrograde axoplasmic transport of NGF determined 1 day (-----) and 4 days (———) after capsaicin treatment.-- Quantity of ^{125}I-NGF in dorsal root ganglia was determined 24 hours after unilateral injection of ^{125}I-NGF (1 uCi) into a front footpad. Values are mean + S.E.M. N=5 animals/point. *p < 0.05 vs. vehicle-treated control value by ANOVA and Scheffe's test.

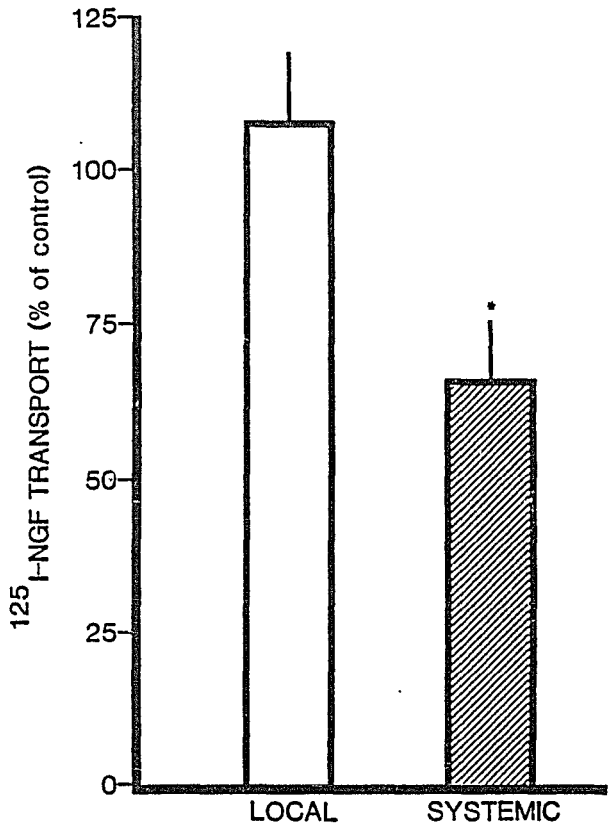


Figure 36. Effect of local administration of capsaicin (8 ug) into a footpad 4 days prior to injection of ^{125}I-NGF at the same site.-- Systemically administered capsaicin (50 mg/kg s.c.) is included as a positive control. N= 5 animals/ group. *$p < 0.05$ by ANOVA and Scheffe's test.

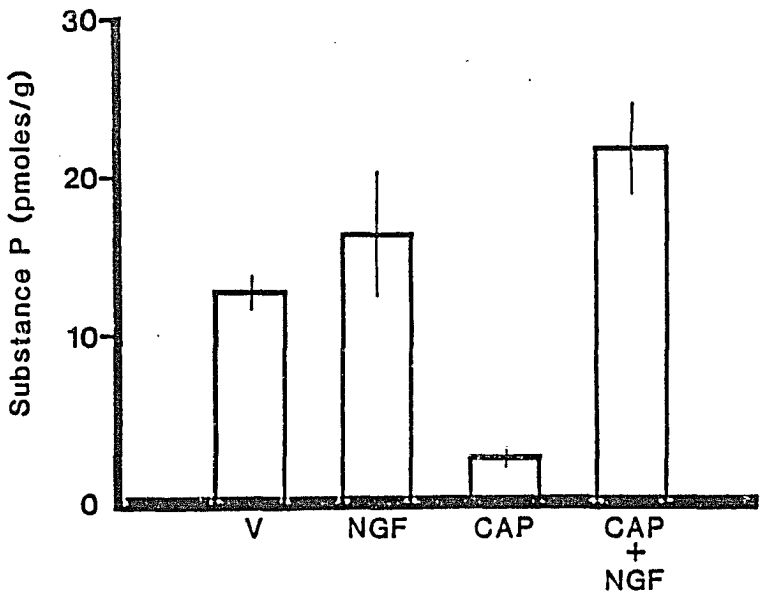


Figure 37. Effect of capsaicin (CAP)(10 mg/kg s.c.) on substance P content of dorsal root ganglia in Hartley guinea pigs supplemented with mouse NGF (4 x 1.0 mg/kg/day).-- Values were determined 4 days after capsaicin treatment. NGF treatment alone produced no statistically significant increase in dorsal root ganglia substance P content. Capsaicin alone markedly depleted substance P ($p < 0.05$ vs vehicle-treated by ANOVA and Scheffe's test) and NGF supplementation prevented capsaicin-induced substance P depletion ($p < 0.001$ for interaction by 2-way ANOVA). Values are mean $\pm$ S.E.M. N=4 in NGF treated groups and 6 in all other groups. Similar results were obtained in a subsequent experiment using a second preparation of NGF.

capsaicin-treated animals. Administration of NGF alone produced no statistically significant change in dorsal root ganglia substance P content (Fig. 37). Furthermore, NGF administration did not prevent capsaicin-induced alterations in response to zingerone.

DISCUSSION

Capsaicin, the primary pungent component of hot peppers, is capable of producing a variety of effects on biological systems. As previously stated, the effects of capsaicin on neuronal tissue are the most profound. In this study, guinea pigs which were treated with a single dose of capsaicin (50 mg/kg s.c.) demonstrated chemogenic and thermal antinociception. The antinociception induced by capsaicin appeared somewhat specific in that responses to mechanical distortion, pressure, and cold were not affected by capsaicin treatment.

It has been suggested for some time that responses to chemical and thermal (heat) stimuli are mediated by slow conducting unmyelinated C fibers, while other sensory responses utilized larger diameter myelinated A delta fibers (Iggo, 1974). A logical inference is that capsaicin specifically alters the ability of C fibers to respond to nociceptive stimuli. Capsaicin could accomplish this in several ways. First, capsaicin could simply cause C fibers to die. This is probably not the case, since neuronal degeneration has been not reported in adult animals treated with capsaicin (Scadding, 1980). Second, capsaicin could alter the ability of C fibers to conduct action potentials. This postulate is also unsupported since electrophysiologic studies have clearly demonstrated that C fibers in capsaicin-treated animals will conduct action potentials following electrical stimulation (Porszasz and Jancso, 1957). This leads to the third potential mechanism by which capsaicin may alter C fiber

function, which is an alteration of the ability of C fibers to generate action potentials. Data supporting this postulate comes from electrophysiological studies by Porszasz and Jancso (1957) in which it was shown that capsaicin pretreatment blocked the initiation of evoked potentials in response to peripheral chemogenic stimulation. Hence, it appears that capsaicin may alter nociception by an action on the distal ends of C fibers.

Following the discovery by Jessell et al. (1978) that capsaicin depleted the putative peptide neurotransmitter, substance P, from primary afferent neurons, the emphasis in capsaicin-related research shifted. It was then speculated that capsaicin induced antinociception by depleting the putative neurotransmitter substance P. The implication was that primary afferent neurons in capsaicin-treated animals functioned to the point of synaptic transmission. However, transmission of nociceptive information failed due to depletion of the neurotransmitter. Many investigators, however, failed to view depletion of substance P by capsaicin as crucial in light of the electrophysiologic work of Porszasz and Jancso (1957). Whether capsaicin-induced depletion of substance P was secondary to the inhibition of action potential generated at the distal ends of sensory neurons is not known. Conversely, the possibility that depletion of the putative transmitter substance P could in some manner inhibit action potential generation has yet to be explored. Insight into these suggestions could be gained in several ways. Of paramount importance is the tissue specificity with which capsaicin depletes substance P. This was accomplished in this study simply by determining the effect of

capsaicin on substance P levels of neuronal tissues which do not contain primary afferent neurons. Towards this end, substance P was measured in hypothalamus, corpus striatum and ventral spinal cord of guinea pigs treated with capsaicin. Capsaicin treatment did not alter substance P in these tissues which presumably contained no primary afferent neurons. Yet capsaicin readily depleted substance P from dorsal root ganglia and dorsal spinal cord. These data suggest that, in relation to the factors involved in the regulation of substance P content, sensory neurons are very different from other substance P containing neurons. However, the data provide us with little insight as to the importance of substance P depletion.

The specificity with which capsaicin depleted substance P from sensory neurons was then investigated. In addition to substance P, the amino acid glutamate is thought to possibly serve as a nociceptive neurotransmitter in primary afferent neurons (Johnson, 1972; Curtis and Johnson, 1974). Measurement of glutamate content in dorsal spinal cords of animals treated with capsaicin revealed no detectable alteration of glutamate levels (Table 5). Yet substance P levels were markedly depleted (Table 4). These data are important because they demonstrate that of the two compounds which are thought by many to be primary afferent neurotransmitters, only one is affected by capsaicin. Hence, in agreement with the sensory testing data, it is apparent that capsaicin does not alter the biochemistry of all sensory neurons. In fact, the suggestion is that capsaicin alters only the peptidergic neurons.

Data regarding the action of capsaicin on primary afferent neuropeptides, other than substance P, has recently become available. Buck et al. (in press) demonstrated that capsaicin treatment did not alter levels of neurotensin, angiotensin II, cholecystokinin or somatostatin in guinea pig dorsal root ganglia. These neuropeptides, though not in the same neurons as substance P, are found in C fibers which are similiar to those which contain substance P (Hokfelt et al., 1976). Hence, again it appears that capsaicin specifically alters the pharmacodynamics of substance P.

The levels of substance P in sensory neurons could be altered by capsaicin in several ways. Capsaicin could inhibit the synthesis of substance P, alter the axoplasmic transport of substance P, alter the storage of substance P, or alter the release of substance P. Since capsaicin depletes substance P from both the dorsal root ganglia, which is the site of substance P synthesis, and the dorsal spinal cord, which is the site at which substance P may serve as a neurotransmitter, alterations in axoplasmic transport of substance P appears unlikely. Decreased transport of substance P would result in increased concentrations of substance P at the site of synthesis. Since this was not observed, emphasis must be placed on the effect of capsaicin on the synthesis, storage or release of substance P.

The effect of capsaicin on the synthesis of substance P was investigated by measuring the incorporation of radiolabelled proline into substance P in vivo. It is important to comment that the study was conducted in vivo so as to rule out potential, and possibly significant, non-specific actions which may result when any chemical

irritant is applied directly to nervous tissue. Results indicated that pretreatment with capsaicin reduced the rate at which radiolabelled proline was incorporated into substance P in dorsal root ganglia by approximately 48 percent ($p < 0.001$). These data indicated that capsaicin depletes substance P from primary afferent neurons by inhibiting the synthesis of substance P. The data must, however, be viewed with some degree of caution because it is not possible to confirm that the relavent pool of proline was uniformly labelled with radiolabelled proline in vehicle and capsaicin-treated guinea pigs.

To establish the specificity with which capsaicin alters substance P synthesis, the effect of capsaicin on total protein synthesis in dorsal root ganglia was investigated. This was accomplished by assessing the incorporation of radiolabelled tryptophan into total protein. No effect of capsaicin pretreatment on the rate of total prtotein synthesis was detected. These data must again be viewed carefully as the uniformity with which the relavent tryptophan pool was radiolabelled was not determined. Furthermore, the extent to which protein synthesis in non-neuronal glia cells contributed to the rates of total protein synthesis was not determined. However, given uniform radiolabelling of amino acid pools and a constant contribution from glial elements, it may be concluded that capsaicin inhibits the synthesis of substance P with at least some degree of specificity. Furthermore, the specificity with which capsaicin affected substance P suggests that alterations in substance P content could be intimately involved in the modality specific antinociception produced by capsaicin.

The temporal relationship between depletion of substance P and the initiation of antinociception was then investigated. The rationale in this experiment was that alterations in primary afferent substance P content preceeding or occurring simultaneously with alterations in nociception would implicate alterations in substance P as the physiologically important biochemical event in capsaicin-induced antinociception. The results, however, clearly demonstrated that antinociception preceeded substance P depletion. These data force one to conclude that at least the initiation of capsaicin-induced antinociception is not a result of depletion of the putative neurotransmitter, substance P, and that substance P depletion and antinociception are possibly separate, dissociable biochemical events. Furthermore, the data indicate that animals must be exposed to capsaicin for at least 24 hours prior to detectable depletion of substance P. This, in itself, suggests that substance P depletion by capsaicin may be a result of a series of biochemical events.

In an attempt to dissociate substance P depletion by capsaicin from antinociception, the pharmacology of a series of capsaicin analogs (Fig. 7) was investigated. In every case, compounds which produced antinociception also depleted substance P from primary afferent neurons. Although only a limited number of capsaicin analogs were tested, the data suggest that the portions of the capsaicin molecule which are necessary to produce antinociception are also required for substance P depletion. For biological activity it appears that both the aliphatic and aromatic moieties of the capsaicin molecule are required. Slight changes in the aliphatic portion, such as

hydrogenation, do not appear to affect activity. This suggests that tritiated dihydrocapsaicin prepared by reducing capsaicin with tritium gas may be employed as a tool for investigating the biochemical mechanisms by which capsaicin acts. To confirm this the pharmacology of dihydrocapsaicin needed to be investigated further. The action of both dihydrocapsacin and capsaicin were assessed on thermoregulatory function and sensory physiology in the rat. Dihydrocapsaicin, which was somewhat more effective than capsaicin, produced hypothermia following parenteral administration which was indistinguishable from that produced by capsaicin in both time of onset and duration. Furthermore, desensitization to the hypothermic effects of dihydrocapsaicin was identical to that seen with capsaicin. Cross-tolerance was observed between capsaicin and dihydrocapsaicin. These data suggests that identical sites of action and mechanisms are involved in the induction of hypothermia.

With regard to sensory systems, both dihydrocapsaicin and capsaicin induced chemogenic antinociception and depletion of substance P from dorsal root ganglia and dorsal spinal cord. Similiar to capsaicin, dihydrocapsaicin did not markedly alter thermal nociception as measured by determining tail-flick latencies. These data indicate that dihydrocapsaicin, or radiolabelled dihydrocapsaicin, are useful tools for investigating the biochemical mechanisms by which capsaicin produces hypothermia, primary afferent dysfunction and depletion of substance P.

To gain insight into the role that metabolism of capsaicin may play in the actions of capsaicin on sensory systems several studies

were conducted. Capsaicin was found to interact with hepatic drug metabolizing systems in a high affinity, concentration dependent manner. This high affinity interaction suggests that capaicin, and its analogs, may be potent inhibitors of biotransformations systems. This postulate is supported by the profound inhibition of ethylmorphine demethylation which was induced by capsaicin in in vitro microsomal incubations as well as by the prolongation of pentobarbital sleeping time seen after in vivo administration of capsaicin. Since capsaicin inhibited ethylmorphine demethylation in vitro in a manner which was not purely competitive, it was not possible to precisely calculate a K_I value. However, capsaicin at a concentration of 25 uM inhibited ethylmorphine demethylation to approximately the same extent as did equimolar SKF 525A. This suggests that capsaicin is as potent an inhibitor of microsomal cytochrome P-450 mediated biotransformation reactions as is SKF 525A. The pharmacological significance of the potent inhibition of biotransformation produced by capsaicin is currenty unknown. However, the potential for altered biotransformation of therapeutic agents as a result of dietary capsaicin should be considered.

Data obtained with ^{3}H-dihydrocapsaicin suggests that capsaicinioids are bioactivated to a species which is capable of covalently interacting with cellular macromolecules. This may explain, at least in part, the partially noncompetitive manner in which capsaicin inhibits ethylmorphine demethylation. Thin layer chromatograpghy data obtained for the metabolites of capsaicin and ^{3}H-dihydrocapsaicin are consistent with the finding of Lee and Kumar

(1980) who demonstrated that capsaicinoids are biotransformed in vitro solely by ring hydroxylation to produce N-(4,5-dihydroxyl-3-methoxy-benzyl)-acylamides. The apparent covalent interaction of dihydro-capsaicin with microsomal protein suggests that hydroxylation of capsaicinoids proceeds through a reactive intermediate, most likely an epoxide (Fig. 38). The inhibition of ^{3}H-dihydrocapsaicin binding to microsomal protein observed in the presence of reduced glutathione further supports the existence of a reactive metabolite of capsaicinoids. The significance of covalent binding of capsaicinoids to hepatic protein has yet to by established. However, detection of radioactivity bound covalently (i.e. non-extractable) to hepatic protein following administration of ^{3}H-dihydrocapsaicin in vivo suggests that hepatic damage reported after long term hot pepper consumption (Lee, 1963a,b) may result from covalent interactions between capsaicinoids and hepatic macromolecules.

Studies dealing with the distrubution of ^{3}H-dihydrocapsaicin demonstrated that radioactivity readily gains access to the brain, presumably including the hypothalamus and corpus striatum, after parenteral administration. Hence, the apparent resistance of hypothalamic and striatal substance P containing neurons to the actions of capsaicinoids appears not to be a result of differential distribution. Finally, the absence of detectable covalent binding of dihydrocapsaicin to protein in brain or spinal cord strongly suggests that the mechanism by which capsaicinoids deplete substance P from the spinal cord does not involve the bioactivation and covalent binding of capsaicinoids to neuronal protein. The role that covalent binding of

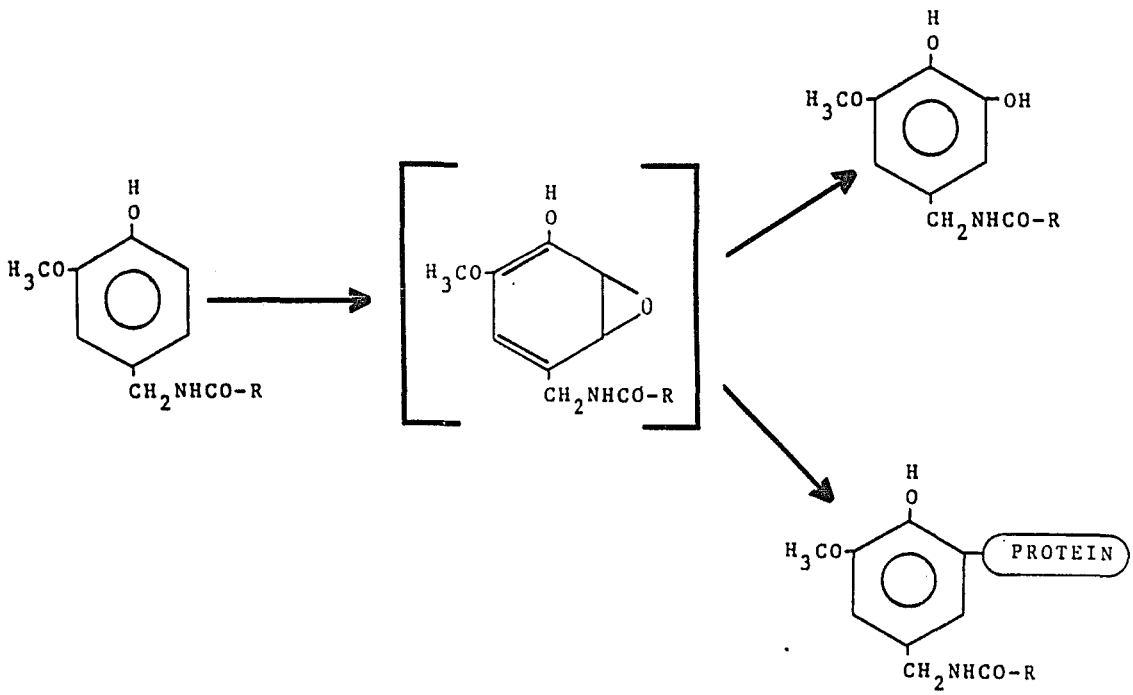


Figure 38. Postulated route of capsaicinoid biotransformation.

capsaicinoids to the distal ends of sensory nerves plays in the etiology of antinociception has yet to be established.

The site at which capsaicin acted to produce antinociception was investigated in guinea pigs which were injected with ^{3}H-dihydrocapsaicin in the right front footpad. The rationale for this experiment was that if capsaicin produced antinociception through an action at the distal ends of sensory neurons then local administration of capsaicinoids at the distal afferent nerve endings should result in antinociception. Data revealed that local injection of dihydrocapsaicin produced antinociception of extremely long duration (> 10 days) but not substance P depletion from dorsal root ganglia. Furthermore, it was found that concentrations of dihydrocapsaicin equivalents were identical in dorsal root ganglia from the ipsilateral analgesic side and contralateral non-analgesic side of the corresponding dermatome. If capsaicin acted at a site in dorsal root ganglia to produce antinociception then in the presence of identical concentrations of dihydrocapsaicin equivalents in ipsilateral and contralateral dorsal root ganglia, both sides would demonstrate antinociception. This was not the case. Thus, it is concluded that capsaicin does not act at a site in dorsal root ganglia to produce antinociception.

The local anesthetic effect of capsaicin was assessed by determining the concentrations of dihydrocapsaicin equivalents in analgesic and non-analgesic skin. Since no direct temporal relationship was observed between levels of dihydrocapsaicin equivalents in skin and the duration of antinociception it is concluded

that the continued presence of free capsaicin is not required for antinociception. Hence, capsaicin does not produce a local anesthetic effect.

The long lasting antinociception produced by capsaicin suggests that capsaicin acts by inducing a very long lasting biochemical alteration. This immediately brings to mind the ability of capsaicin to covalently bind to macromolecules following bioactivation. For this reason, covalent binding of dihydrocapsaicin was assessed in skin from guinea pig feet following local administration. Unextractable dihydrocapsaicin equivalents (ie. presumably covalently bound) in analgesic skin were 3-4 fold greater than levels in non-analgesic skin at all times measured. These data suggest that capsaicinoids may induce antinociception by binding covalently to dermal constituents, possibly free nerve endings. One may speculate that the apparent specificity that capsaicinoids demonstrate for altering chemogenic and thermal nociception may result from an inability of capsaicinoids, or capsaicinoid metabolites, to gain access to encapsulated primary afferent endings, whereas access to free nerve endings is unimpeded.

The mechanism by which capsaicinoids deplete substance P from primary afferent neurons, although apparently unrelated to the induction of antinociception, is of definite pharmacologic interest. Recent studies have demonstrated that capsaicin depletes substance P from primary afferent neurons in the adult guinea pig but leaves somatostatin content intact (Burks et al., 1981). This apparent specificity for depletion of substance P suggests that capsaicinoids may interfere with the action of specific neurotrophic substances.

Sectioning of the peripheral processes of primary afferents (Jessell et al., 1979) or exposure of adult animals to antibodies to NGF (Schwartz et al., 1979) results in depletion of substance P from primary afferents. Hence, capsaicinoids may deplete substance P from afferent neurons by altering the retrograde axoplasmic transport of substances functionally similiar to NGF.

To test this hypothesis the effect of capsaicin on retrograde axoplasmic transport of NGF was measured. It was found that a single dose of capsaicin which depleted substance P from dorsal root ganglia (50 mg/kg s.c.) also markedly inhibited the retrograde axoplasmic transport of NGF. In vehicle and capsaicin-treated animals, NGF was detected in dorsal root ganglia within 4 hours and maximum concentrations were attained 24 hours after peripheral injection. The maximum concentration of ^{125}I-NGF in dorsal root ganglia of capsaicin-treated animals was approximately 45 percent of that observed in vehicle-injected control animals ($p < 0.05$ by ANOVA). These data indicate that capsaicin inhibits the quantity of NGF being transported but not the rate of retrograde transport. Decreases in the quantity of NGF transported could result from either a diminished number of neurons transporting NGF or a decrease in the uptake of NGF at peripheral afferent terminals. To what extend either of these mechanisms are involved in the actions of capsaicin is currently unknown.

Investigating the dose-response relationship for inhibition of retrograde axoplasmic transport of NGF by capsaicin revealed that doses of capsaicin which depleted substance P also inhibited the retrograde axoplasmic transport of NGF. Furthermore, alterations in NGF transport

preceeded substance P depletion. These data are consistent with the hypothesis in which capsaicin depletes substance P by preventing NGF from reaching the perikaryon of sensory neurons via retrograde axoplasmic transport.

To further test this hypothesis, the effect of locally applied capsaicin on retrograde axoplasmic transport of NGF was determined. Locally administered capsaicin produces antinociception but not substance P depletion. The experiment was designed to assess the relationship between capsaicin-induced antinociception and capsaicin-induced inhibition of retrograde axoplasmic transport. Data revealed that locally administered capsaicin did not alter NGF transport. Hence, alterations in the retrograde axoplasmic transport of NGF appear to be associated with substance P depletion and not antinociception. Furthermore, these data demonstrate that capsaicin does not inhibit NGF transport by a primary action on the distal ends of sensory neurons resulting in an inhibition of NGF uptake. It appears that, like substance P depletion, capsaicin must act at a site more central than the distal ends of sensory neurons to inhibit the retrograde axoplasmic transport of NGF.

The cause and effect relationship between depletion of substance P and alterations in the retrograde axoplasmic transport of NGF was investigated in guinea pigs which were treated with capsaicin and supplemented with mouse NGF. Systemically administered NGF could reach the cell bodies of primary afferent neurons directly through the systemic circulation and would not be dependent upon retrograde axoplasmic transport (Johnson, 1978). Data revealed that

administration of NGF completely blocked capsaicin-induced depletion of substance P. Administration of NGF alone produced no significant change in dorsal root ganglia substance P content. Furthermore, NGF administration did not prevent capsaicin-induced alterations in response to the chemical irritant zingerone. These data indicate that capsaicin-induced depletion of substance P is a result of decreased availibility of NGF to sensory neurons as a result of alterations in retrograde axoplasmic transport. Also, the data conclusively show that capsaicin-induced antinociception is completely unrelated to depletion of substance P.

Finally, in addition to describing the biochemical mechanisms by which capsaicin depletes substance P from primary afferent neurons, the data suggest that endogenous NGF is required for the maintenance of the normal neurochemistry of at least one group of sensory neurons in adult animals. By analogy, one may speculate that other neuropeptidergic systems, such as opiates (endorphins and enkephalins), neurotensin, cholecystokinin, somatostatin, vasoactive intestinal peptide and angiotensin II, may be regulated in a similiar manner. Thus, effects on the pharmacodynamics of neurotrophic factors, such as NGF, represent a novel mechanism by which pharmacological and toxicological agent may modify neuronal function and deserves to be the subject of intensive future study.

CONCLUSIONS

1. Capsaicin administration to guinea pigs results in a dose-dependent (2-8 mg/kg s.c.) chemogenic and thermal (heat) antinociception. Antinociception occurred within 2 hours and lasted in excess of 10 days.

2. Capsaicin administration results in a dose-dependent (4-25 mg/kg s.c.) depletion of substance P from primary afferent neurons.

3. Depletion of substance P by capsaicin is not the mechanism by which capsaicin produces antinociception.

4. Capsaicin-induced antinociception may be a result of bioactivation and covalent binding of capsaicin to the distal ends of sensory nerves.

5. One mechanism by which capsaicin depletes substance P is by inhibiting the synthesis of substance P.

6. Capsaicin-induced depletion of substance P is a result of alterations in the retrograde axoplasmic transport of an endogenous nerve growth factor-like substance.

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