MODULATION OF I_h IN PRIMARY AFFERENT NEURONS BY PROSTAGLANDIN E2 AND OPIOIDS

by

Susan L. Ingram

A DISSERTATION

Presented to the Department of Pharmacology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

September 1995

APPROVED:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. John T. Williams, for sharing his time, knowledge and constructive criticism. I learned from John that science can be a challenging, rewarding and fun career. His positive attitude in the lab is one characteristic that I truly admire and will strive to achieve throughout my career.

I would also like to thank the members of my thesis advisory committee, Dr. Steve Johnson, Dr. Ed McCleskey and Dr. Gary Westbrook, for their advice and constructive criticism on the work presented in the thesis.

I would like to thank Ken Drews for preparing the cultures of the primary afferent neurons and Zach Hausken for help in interpreting data with modulators of protein kinase A. The members of the Williams lab during the last three years also deserve a special thanks for making the Williams Lab a great place to learn and appreciate science: Don Cameron, Peregrine Osborne, Sharon Oleskevich, Tim Grudt, Masaru Ishimatsu, Antonello Bonci and Chris Fiorillo.

I would also like to thank the many people in the Vollum Institute and the Departments of Pharmacology and Neuroscience past and present who have helped to shape and stimulate my interest in science. In particular I would like to thank Drs. Neil Marrion, Julie Saugstad, Andreas Busch, Vladimir Gerzanich, Don Cameron, Marcelo Rubenstein, Armando LaGrutta and Melissa Martenson. Special thanks are extended to the graduate students who also began graduate careers at OHSU in the fall of 1990 and have become my closest friends, Anne Burleigh, Shelly Dickinson, Sunita DeSouza, Laura Kozell and Sarah Coste.

There are several people that were not directly associated with my life as a graduate student, but have played important roles in my life and without whom I would not be at this point in my career. My experience in Portland would certainly have not been the same or a

satisfying without sharing it with my best friends Crystal and William Gwyn. My special thanks to Matthew Osborn for his unconditional love, support and patience. I will never be able to express how much it has meant to me to have been able to share his life and family during the past three years.

I am also greatly indebted to my parents, Steven and Shirley Ingram, for their guidance and encouragement throughout my life and allowing me to continue my education so far away from home. I also thank them for teaching me that I can achieve anything I want with hard work. I want them to know that I realize that I am extremely lucky to have such a supportive family... no one could ask for more.

ABSTRACT

Peripheral tissue damage and inflammation produce pain and hyperalgesia. Substances released into the periphery during the inflammatory response cause excitation and sensitization of primary afferents, but the mechanisms of this activation are not known. Hyperalgesia and inflammation are also associated with increased levels of intracellular cAMP. Alternatively, peripheral administration of opioids causes analgesia, reduces excitability of small-diameter primary afferents and decreases cAMP levels during inflammation. Opioid receptors are coupled to K^+ channels, Ca^{+2} channels and adenylyl cyclase; however, the mechanisms of opioid inhibition of peripheral pain are unknown. Because excitation of neurons is associated with ion channel activity, we chose to study the effects of PGE2 and opioids on I_h, a hyperpolarization-activated cation channel that is regulated by cAMP.

Whole-cell recordings from nodose ganglion neurons in culture determined that primary afferent neurons express a hyperpolarization-activated inward current (I_h) that is sensitive to changes in cAMP concentration. PGE2, forskolin and cAMP analogs shifted the voltage dependence of I_h to more depolarized potentials. Although μ-opioids had no effect on I_h in control conditions, they inhibited forskolin-stimulated I_h in a naloxone-sensitive manner in a subpopulation of nodose neurons. Opioid inhibition was not effective in the presence of cAMP analogs suggesting that the mechanism of opioid inhibition is inhibition of adenylyl cyclase.

Several studies were done to further characterize the subpopulation of nodose neurons which respond to opioids. Intracellular recordings from an intact ganglion preparation confirmed observations by other groups that the nodose ganglion contains neurons with conduction velocities in the Aδ- and C-fiber range. Furthermore, these studies determined that I_h was expressed only in neurons with faster conduction velocities (Aδ-cells).

Another set of experiments was designed to determine the mechanism by which PGE2 and forskolin shift the activation curve and increase the maximum amplitude of I_h. The cAMP analogs, RP-cAMP-S (an inhibitor of protein kinase A) and SP-cAMP-S (an activator of protein kinase A), mimicked the effects of PGE2 and forskolin. The inhibitor peptide of protein kinase A, the catalytic subunit of protein kinase A, and phosphatase inhibitors had no effect on PGE2 or forskolin modulation of I_h. These results suggest that I_h may be directly modulated by cAMP and may be related to cyclic nucleotide-gated channels in sensory neurons.

Since tissue damage and inflammation excite primary afferents and I_h has been shown to modulate firing in central neurons, we were interested in determining if PGE2 and forskolin could modulate firing in peripheral neurons. Whole-cell recordings were made in current-clamp mode from cultured nodose ganglion neurons expressing I_h. PGE2 and forskolin decreased the threshold of activation, depolarized the membrane, and increased the number of action potentials elicited during a supra-threshold stimulus. Therefore, modulation of I_h by increased cAMP levels may be a possible mechanism of chemical pain transduction. Positive regulation of I_h by prostaglandins produced during inflammation may lead to depolarization and facilitation of repetitive activity. On the other hand, opioid inhibition of adenylyl cyclase and subsequent inhibition of I_h may be a mechanism by which opioids inhibit primary afferent excitability and relieve pain.

INTRODUCTION

Pain is defined as the sensory and perceptual experience of tissue damage. Perception of a noxious stimulus as painful is thought to be the mechanism by which an organism is alerted to damage within itself and subsequently protects the injured area. Inflammation seems to have a role in repairing tissue damage. The mechanisms by which tissue damage is transduced into the perception of pain are not completely understood, but these processes have been important areas of research for centuries. Many currently accepted principles of pain perception were defined by anatomists and physiologists by the early 1900's. Nociception is defined as the actual sensation of tissue damage with no contribution of affectional or motivational aspects. Theories of pain have attempted to answer fundamental questions about the transmission and perception of pain on the basis of histological and clinical observations. How does tissue damage elicit pain? Is pain a 'specific' sense: one fiber mediating one sensation? What role do nerve impulses play in the perception of pain? Is the spinal cord a simple relay for pain or does it modify afferent input? Although there have been thousands of studies on pain since the early 1900s, many questions concerning perception of pain and mechanisms of nociception are still unanswered. The focus of this work will be on a possible mechanism of transduction of inflammatory pain via modulation of an ion current.

Early studies correlated excitation of specific primary afferents during tissue damage with the perception of pain. The mechanisms by which tissue damage is transduced into electrical impulses in primary afferents is not known. Furthermore, many groups have studied the mechanisms of pain regulation in the central nervous system, but peripheral modulation has largely been ignored. The Introduction will give a brief overview of the pain pathways and historical background of our current knowledge of pain mechanisms. The second section will discuss central mechanisms of pain modulation with an emphasis on a descending inhibitory pathway, as well as possible mechanisms of modulation at central terminals of primary afferents. The final section of the Introduction will describe the events that occur in the periphery during tissue damage and inflammation and how these events may modulate nociception at the peripheral terminals of primary afferents.

I. HISTORY OF CURRENT THEORIES OF PAIN TRANSMISSION

A. Theories of Pain

Sensations of the skin have been classically divided into four groups (touch, pain, cold and warm) mediated by anatomically separate pathways. This classification formed the foundation of the 'Specificity Theory' of cutaneous sensation based on the Doctrine of Specific Energies of Johannes Muller in the 1830's and the histology of von Frey in the late 1800's who described specific nerve endings associated with 'spots' of specific sensation. Since there was a lack of evidence for specific nerve endings associated with pain sensations and electrophysiological evidence that pain was carried by both myelinated and unmyelinated fibers (Head, Rivers, & Sherren, 1905; Head & Thompson, 1906), it was hypothesized that pain was not considered a specific sensation (Nafe, 1927; Sinclair, 1955; Weddell, 1955). Nafe and colleagues proposed the 'Pattern Theory', that the brain perceived painful stimuli as a characteristic pattern of impulses based on observations that increased stimulus intensities increased both the frequency of nociceptor firing and the duration of the response (Adrian, 1927). However, as experimental techniques advanced, a large amount of receptor-fiber specialization was observed. Thus, a more integrative theory was proposed by Melzack & Wall (1965a) that combined aspects of both the Specificity and Pattern Theories. Their alternative hypothesis was that cutaneous receptors have specialized physiological properties for the transduction of stimuli into patterns of nerve impulses. Central neurons detect details from many inputs through impulse patterns and spatial summation of the inputs. The Gate Control Theory (Melzack & Wall, 1965b), proposed that cells in the substantia gelatinosa modulated afferent information before it was transmitted to the brain. In this theory, three features of afferent input were thought to be important for pain perception: the ongoing activity that precedes noxious stimulation, the stimulus-evoked activity, and the relative balance of activity in large versus small afferent fibers. Therefore, the somesthetic system could act as an integrative system that discriminates between different sensations produced by unique patterns of impulses.

B. Ascending Pain Pathway

1. Nociceptors

Cutaneous pain originates in the skin and is transmitted to the central nervous system via primary afferents. Tissue damage (crushing, pinching, tearing, heating and freezing of the skin) is the natural stimulus for pain. Each stimulus modality has a specialized nociceptive ending (Adrian, Cattell, & Hoagland, 1931; Iggo, 1963; Perl, 1968) that when activated elicits a generator potential that transduces its activation into action potentials within the corresponding primary afferent fiber (Loewenstein, 1963). Loewenstein (1963) found that the generator potential in Pacinian corpuscles, specialized mechanoreceptor endings, was elicited by a conductance increase in the membrane at the point of stimulation. It is currently assumed that proper stimulation of nociceptor endings also elicits a generator potential resulting in activation and transduction of the stimulus, but the mechanisms are not understood.

Nerve stimulation elicits compound action potentials with a nociceptive and an innocuous wave with different conduction velocities (Ranson, 1915). The nociceptive wave results from activation of small-diameter, thinly myelinated (Aδ-) and unmyelinated (C-) afferents (Collins, Nulsen, Randt, 1960; Bessou & Perl, 1969). Aδ- and C-fiber endings are free nerve endings embedded into their target tissues (both skin and deep structures). Aδ-fibers have conduction velocities of approximately 5 - 30 m/s and respond to thermal and mechanical stimuli to produce a sharp, pricking pain. C-fibers have conduction velocities in the range of 0.5 - 2 m/s and respond to high intensity mechanical stimuli, as well as to chemical, noxious heat and cold stimulation. These characteristics correspond to the psychophysical definition of first pain (Aδ-fibers) and second pain (C-fibers) (Sinclair & Stokes, 1964).

The cell bodies of nociceptors are located in ganglia found, in some cases, a great distance from their peripheral targets (Ranson, 1912). The ganglia contain heterogeneous populations of primary afferent neurons that are differentiated by their responses to chemical stimuli, fiber types, cell size, neuropeptide content, action potential

characteristics, membrane properties, etc (Baccaglini & Hogan, 1983; Tokimasa, Tsurusaki, & Akasu, 1983; Harper & Lawson, 1985). Studies classifying primary afferents have been repeated in different ganglia, with different stimuli, and in different species. Consequently, the literature on this subject is immense and has provided little additional insight into the mechanisms of pain. Therefore, only studies which have focused on correlating cell characteristics with functional differences will be cited. One important observation is that many of the *in vivo* characteristics seem to be maintained in culture (Baccaglini & Hogan, 1983), particularly on the cell soma. The function of the primary afferent soma is not known. It may simply be a support system for the transmission of impulses along the axons, or it may modulate afferent input.

The central process from the cell body is sent to synapse within the dorsal horn. The dorsal horn is divided into laminae on the basis of cytoarchitecture (Rexed, 1952). Myelinated and unmyelinated neurons are distributed within the dorsal horn in a highly specific manner. Both types of neurons bifurcate upon entering the spinal cord in the Tract of Lissauer (Ranson, 1913; 1914; Ranson & Billingsley, 1916), synapsing in several different segments. The Aδ-fibers seem to travel for three to five segments rostrocaudally (Giesler, Cannon, Urca & Liebeskind, 1978; Cervero, Iggo & Molony, 1979) and terminate in Laminae I, the substantia gelatinosa (Laminae II and III), V and X. C-fibers are more limited in their extension to different segments and terminate primarily to Laminae I and the substantia gelatinosa. All small-diameter primary afferents terminate within the dorsal horn.

Although it is not clear what neurotransmitters and neuropeptides are released by certain types of primary afferents, glutamate and other excitatory amino acids are prime candidates. A subset of neurons in laminae I and II excited by C-fiber components of dorsal root volleys are excited by iontophoretic glutamate and aspartate (Schneider & Perl, 1985), and a greater concentration of excitatory amino acids are found in the dorsal horn relative to the ventral horn of the spinal cord (Duggan & Johnston, 1970). The effects of glutamate and aspartate are likely a result of their release by primary afferents because smaller dorsal root

ganglion neurons are selectively associated with uptake of glutamate and aspartate (Duce & Keen, 1983; Cangro, Sweetnam, Wrathall, Haser, Curthoys & Neale 1985). In addition, several neuropeptides are associated with primary afferent fibers, such as substance P, calcitonin gene-related peptide and somatostatin (for review, see Holzer, 1988). Intrathecal administration of substance P causes painful responses (Piercey, Dobry, Schroeder, & Einspahr, 1981) and a weak excitation of dorsal horn neurons (Randic & Miletic, 1977; Hentall & Fields, 1983; Stanfield, Nakajima & Yamaguchi, 1985). Substance P is co-localized with glutamate in primary afferents (De Biasi & Rustioni, 1988) and with serotonin in medullo-spinal projection neurons (Barber, Vaughn, Slemmon, Salvaterra, Roberts & Leeman, 1979; Johansson, et al., 1981). Substance P has also been proposed to be the antidromically released neurotransmitter that initiates peripheral hyperalgesia (for review, see Lembeck, 1983). Calcitonin gene-related peptide enhances tachykinin-induced protein extravastion (Gamse & Saria, 1985) and somatostatin inhibits protein extravasation in the periphery (Lembeck, Donnerer & Barthó, 1982), but little is known about their effects in the spinal cord.

2. Spinal Cord Neurons

In Laminae I and the substantia gelatinosa, there are some neurons which respond exclusively to nociceptor activation, either mechanical nociception or polymodal nociception. However, there are also cells which respond to thermal input, innocuous mechanical input, and multi-receptive inputs. Many of the projections of spinal dorsal horn neurons were determined by degeneration of axons after lesions of the spinal cord (Vierck & Luck, 1979) or retrograde labeling with horseradish peroxidase (Willis, Leonard & Kenshalo, 1978; Gieseler, Cannon, Urca & Liebeskind, 1978). Lamina I - III neurons project to a number of rostral brain sites (thalamus, hypothalamus, and the midbrain), as well as locally to the deeper Laminae IV and V. Laminae IV and V receive direct inputs from primary afferents, but these contacts are much more sparse than in Laminae I - III. Lamina V consists of nociceptive, multireceptive, and innocuous mechanoreceptive dorsal horn neurons that provide the majority of ascending projections to the thalamus. The complexity added by convergent inputs to dorsal horn neurons provided fodder for many

more classification papers. Functionally, dorsal horn neurons have been classified as nociceptive-specific (responding to noxious inputs only) or wide-dynamic range neurons (responding to both noxious and innocuous inputs). Wide dynamic range neurons receive noxious inputs from both Aδ- and C-fibers (Mendell & Wall, 1965; Mendell, 1966).

The spinal cord contains several fiber tracts which carry nociceptive information to higher brain centers: the spinothalamic tract, spinoreticular tract, spinomesencephalic tract and the spinocervical tract. The spinothalamic tract originates in Lamina I, V, and VII (Willis, Leonard & Kenshalo, 1978) and includes both wide-dynamic range and nociceptive-specific neuron projections. This tract transmits information from nociceptors, temperature, tactile pressure and proprioception. The spinoreticular tract consists of nociceptor infomation from neurons in Laminae VII and VIII (Fields, Clanton & Anderson, 1977), and is thought to play a role in arousal. The spinomesencephalic tract originates from neurons in both Lamina I and V and sends projections to the mesencephalic reticular formation, the periaqueductal grey and other midbrain sites. The spinocervical tract sends nociceptive information from Laminae III and IV through the dorsolateral spinal cord to the lateral cervical nucleus. There is much crosstalk and many collateral projections throughout all aspects of the ascending pain pathway, adding to its extreme complexity.

C. Descending Inhibition of Pain

Pain can be modulated at different levels of the pain pathway. The focus of this section will be on central mechanisms of inhibition of pain. Sherrington and Sowton (1915) showed that spinal reflexes increase after transection of the spinal cord, indicating the presence of an descending inhibitory control. Many studies have observed differential characteristics of dorsal horn responses depending on whether a decerebrate or spinal preparation was studied (eg., Wall, 1967; Hillman & Wall, 1969). Consequently, descending modulation of nociception has been a major area of research and many areas of the brainstem have been implicated in this control.

1. Descending Inhibitory Pathway from Brainstem

Analgesia has been elicited by electrical stimulation of many brainstem areas, including the hypothalamus (Carstens, 1982; Aimone & Gebhart, 1987; Aimone, Jones & Gebhart, 1988), periaqueductal gray (Aimone, Jones & Gebhart, 1987), locus coeruleus (Hodge, Apkarian, Stevens, Vogelsang & Wisnicki, 1981; Jones & Gebhart, 1986), raphe nuclei (Oliveras, Redjemi, Guilbaud & Besson, 1975), and the nucleus tractus solitariius (Lewis, Baldrighi & Akil, 1987). Naloxone injections into the PAG of humans and rats have been shown to block stimulation-produced antinociception (Adams, 1976; Akil, Mayer & Liebeskind, 1976; Hosobuchi, Adams & Linchitz, 1977). In addition, microinjections of morphine and opioid peptides into the brainstem can elicit antinociception and inhibit dorsal horn responses to noxious stimulation (Yaksh, Yeung & Rudy, 1976; Jensen and Yaksh, 1986) suggesting that opioids are implicated in antinociception in the central nervous system. In this section, the focus will be on the areas that compose the major descending inhibitory pathway: the periaqueductal gray (PAG), nucleus raphe magnus (NRM), and the spinal cord (Basbaum & Fields, 1978; 1984; Fields, Barbaro & Heinricher, 1988) (see Figure 1).

The periaqueductal gray (PAG) is an organized processing center for integration of stimuli (noxious, threatening and stressful) to produce defense responses (see Bandler & Shipley, 1994) and an important site in the descending control of nociception (Basbaum & Fields, 1984; Fields, et al., 1988; Reichling, et al., 1988). The PAG receives afferents from limbic, autonomic, motor and sensory systems, and nociceptive afferents projecting from the spinal cord (Eickhoff, Handwerker, McQueen & Schick, 1978; Pechura & Liu, 1986; Beart, Summers, Stephenson, Cook & Christie, 1990) and projects to the brainstem. The main descending inhibitory efferent projection from the ventrolateral column of the PAG is to the nucleus raphe magnus (NRM) (Yezierski, et al., 1982; Aimone, Jones & Gebhart, 1987; Cameron, Khan, Westlund & Willis, 1995). Stimulation of the PAG has been shown to excite NRM neurons (Behbehani & Fields, 1979; van Praag & Frenk, 1990). The NRM receives projections from both the spinal cord and the PAG (Behbehani & Fields, 1979; Pechura & Liu, 1986) and projects to other areas of the brainstem, as well as

to spinal cord laminae involved in nociceptive transmission (Basbaum, & Fields, 1978; 1986; Bennett & Mayer, 1979). The terminations and neurotransmitters associated with projections from the NRM to the spinal cord have not been well-characterized. However, serotonin antagonist administration in the spinal cord has been shown to block the effects of NRM antinociception, indicating a role for serotonin projections in inhibition of pain transmission (Aimone, et al., 1987).

a. Stimulation-produced analgesia

Electrical stimulation of the PAG increases nociceptive thresholds of behavioral measures of pain (eg., Mayer & Liebeskind, 1974; Hosobuchi, et al., 1977) and inhibits activity of dorsal horn neurons responding to noxious stimuli (Bennett & Mayer, 1979). Analgesia produced by stimulation of different PAG areas has different qualities; ventral stimulation often produces profound analgesia, while dorsal and dorsolateral PAG stimulation produce a lesser degree of analgesia concomitant with strong aversive behavioral reactions (Fardin, Oliveras & Besson, 1984).

Electrical stimulation of the NRM and associated areas inhibits nociceptive transmission and reflexes (Balagura and Ralph, 1973; Oliveras, et al., 1975; Fang, Haws, Drasner, Williamson & Fields, 1987). Responses of two types of NRM cells have been linked to stimulation-produced analgesia in the NRM. These two types of cells have been defined on the basis of their responses during the tail-flick nociception test. The tail-flick test entails applying a noxious stimulus to the tail of a rat (usually heat) and measuring the time it takes for the rat to remove its tail. During the noxious stimulus, ON-cells precede the tail-flick response with a burst of firing, while OFF-cells are inhibited. Direct stimulation of the NRM has been shown to elicit continuous firing in OFF-cells and simultaneously inhibit the tail-flick response (Fields, Vanegas, Hentall & Zorman, 1983). Therefore, OFF-cell firing has been linked to inhibition of the tail-flick response to noxious stimuli. On the other hand, ON-cells are inhibited during NRM stimulation and inhibition of the tail-flick response suggesting that they are either NRM interneurons or they mediate excitation of motor neurons in the spinal cord.

b. Opioid analgesia

Opioids and opioid receptors have been localized to the PAG and the NRM (eg., Atweh & Kuhar, 1977; Waksmen, Hamel, Fournié-Zaluski & Roques, 1986). Microinjections of opioid peptides or morphine into the PAG (eg., Jensen & Yaksh, 1986; Fang, Haws, Drasner, Williamson & Fields, 1989) or NRM (Jensen & Yaksh, 1989; Tseng, Tang, Stackman, Camara & Fujimoto, 1990) are associated with inhibition of both spinal neurons and pain-related behaviors (Bennett & Mayer, 1979). $[Met]^5$ enkephalin and morphine hyperpolarize and inhibit spontaneous firing in a subpopulation of PAG neurons, and these actions have been shown to be blocked by naloxone and μ-antagonists (Smith, Robertson, Monroe, Taylor, Leedham & Cabral, 1992). As with stimulation-produced antinociception, morphine antinociception has been shown to be most efficacious in the ventrolateral aspect of the PAG (Yaksh, Yeung & Rudy, 1976). However, the most efficacious loci for stimulation-produced and morphine analgesia in the ventrolateral PAG were not the same (Lewis & Gebhart, 1977) suggesting the presence of a non-opioid descending inhibitory pathway.

Morphine microinjections into the rostral ventromedial medulla (including the NRM) have been shown to increase pain thresholds (Jensen & Yaksh, 1989), and this effect can be blocked by injections of lidocaine in the area of the NRM (Urban & Smith, 1994). Studies looking at the effects of electrical stimulation and morphine injections into the NRM on the excitability of dorsal horn neurons have produced conflicting results. There are reports of inhibition of dorsal horn responses during electrical stimulation of the NRM (Light, Casale & Menetrey, 1986), but morphine injections in the NRM have been shown to facilitate excitation of dorsal horn neurons (Le Bars, Dickenson & Besson, 1980). These contrasting results may be attributed to different stimulation techniques or stimulation of different areas within the NRM. However, these studies are more likely measuring responses of different populations of dorsal horn neurons. The main behavioral result of both stimulation and morphine injections in the NRM is an inhibition of nociception;

therefore, it is likely that the results of LeBars, Dickenson & Besson (1980) and Light, Casale & Menetrey (1986) were recorded from different populations of neurons within the spinal cord.

2. The Disinhibition Model of Opioid Action

The most widely recognized model of descending antinociception is the disinhibition model of Fields and colleagues (Basbaum & Fields, 1978; 1984). The disinhibition model of opioid action is based on observations that opioids inhibit GABA interneurons (Nicoll, Alger & Jahr, 1980). Opioid suppression of tonic inhibitory inputs from GABA interneurons has been shown to be a major mechanism of opioid-induced excitation in many areas of the CNS (eg., Pan, Williams & Osborne, 1990; Cohen, et al., 1992; Johnson & North, 1992). GABA antagonists have been shown to excite neurons in the PAG and NRM (Lin, Peng & Willis, 1994). This indirect excitation is responsible for the ultimate inhibition of nociceptive processing in the spinal cord (Behbehani & Fields, 1979) (see Figure 1).

There is substantial indirect data which supports the disinhibition hypothesis in the PAG and the NRM. Opioid receptors and peptides are abundant in the PAG and the NRM (Atweh & Kuhar, 1977; Waksman, et al., 1986; Mansour, Khatchaturian, Lewis, Akil & Watson, 1987). Enkephalin immunoreactive terminals form synaptic contacts with GABA immunoreactive neurons in the PAG (Wang, et al., 1994). Retrograde tracing from the medulla to the PAG and immunocytochemistry studies have confirmed that there are abundant connections between GABA cells and output PAG neurons (Reichling & Basbaum, 1990 a,b). PAG projection neurons are not GABAergic and are presumably excitatory (Barbaresi & Manfrini, 1988). Microinjections of $GABA_A$ agonists into the PAG inhibit morphine-induced analgesia while GABA antagonists potentiate the analgesic effect of morphine (Zambotti, et al., 1982; Moreau & Fields, 1986; Depaulis, Morgan & Liebeskind, 1987). Although intracellular recordings have shown that opioids can inhibit a GABA-mediated synaptic potential in a subset of PAG neurons, they also produce similar inhibition of glutamate synaptic potentials (Chieng & Christie, 1994a) and directly

hyperpolarize some PAG neurons (Behbehani, Jiang & Chandler, 1990; Chieng & Christie, 1994b). Thus, disinhibition may not be the only mechanism of descending inhibition to the NRM from the PAG.

There is more direct data supporting the disinhibition hypothesis in the NRM. Morphine microinjections affect firing properties of some NRM neurons providing direct support for the descending inhibitory pathway (PAG-NRM-spinal cord). Similar to results during stimulation of the NRM, morphine produces opposing actions in two physiologically different NRM neuron subtypes as defined by the tail-flick reflex (Heinricher, Cheng & Fields, 1987; Fang, et al., 1989; Morgan, Heinricher & Fields, 1992). Cells that precede the tail-flick with a suppression of firing (OFF-cells) were excited, but cells that increase activity just before and during the tail-flick (ON-cells) were variably depressed in the presence of morphine (Fields, et al., 1983). These results are mimicked by iontophoresis of morphine onto ON-cells, but not OFF-cells suggesting that morphine excitation of OFF-cells is not direct (Heinricher, Morgan, Tortorici & Fields, 1994). Thus, OFF-cells have been hypothesized to be spinal cord projection neurons tonically inhibited by GABA input, and ON-cells may be GABA interneurons. In support of this hypothesis, bicuculline (a GABA antagonist) injections into the PAG or NRM have been shown to excite OFF-cells and to inhibit the tail-flick response (Heinricher & Tortorici, 1994), but have no effect on ON-cells. GABA immunocytochemistry studies have localized GABA-containing neurons to the NRM, and retrograde labeling studies from the spinal cord have determined that there are no GABA NRM-spinal cord projection neurons (Reichling & Basbaum, 1990). Thus, opioid inhibition of GABA inputs to NRM-spinal projection neurons may be the mechanism of NRM descending inhibition.

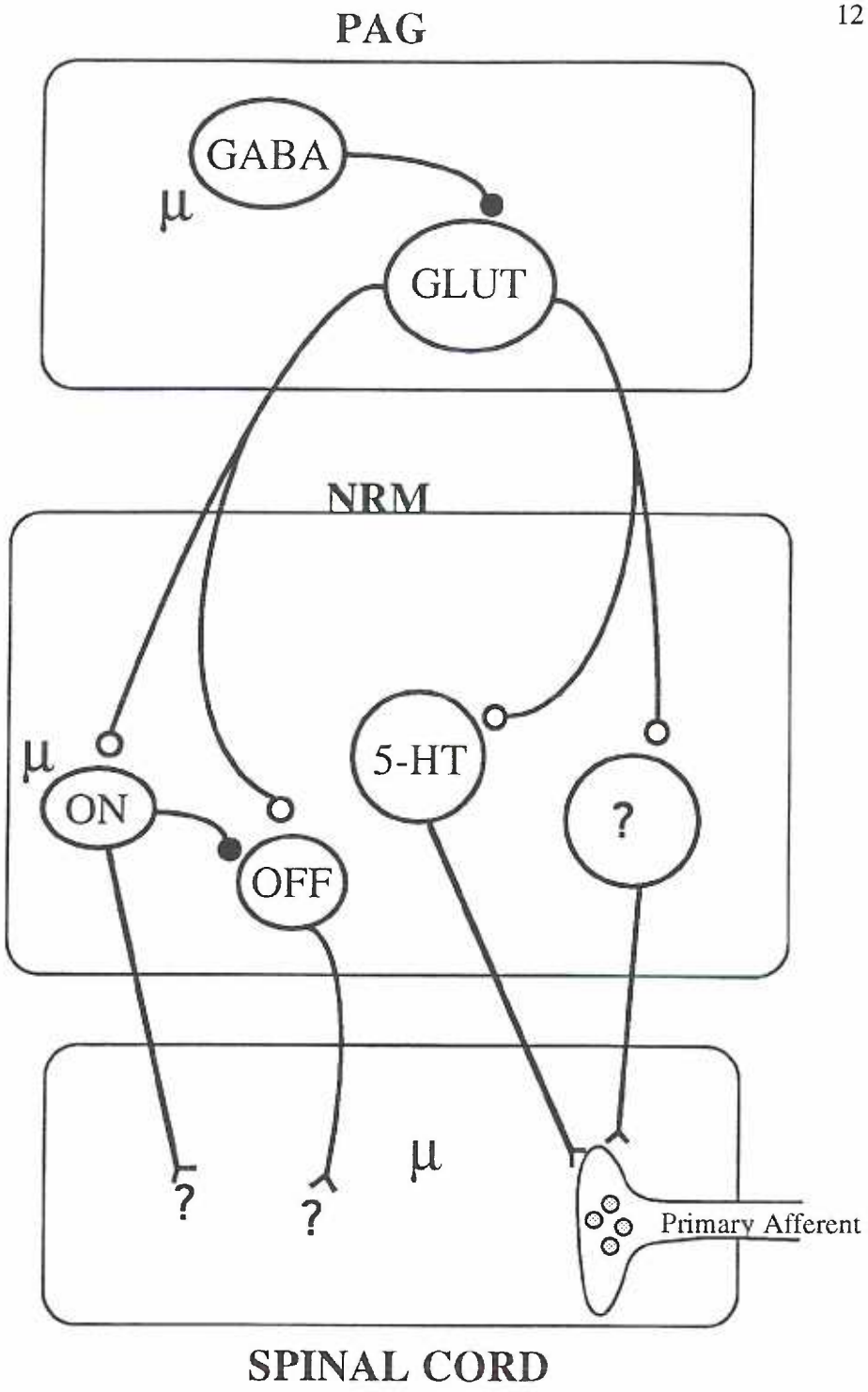

Figure 1. Disinhibition Hypothesis of Descending Inhibition of Pain. Neurons have either excitatory (○) or inhibitory inputs (●). Opioids (μ) inhibit inhibitory interneurons in the periaqueductal gray (PAG) and nucleus raphe magnus (NRM). (GLUT=glutamate)

Dorsal horn neurons are both excited and inhibited by stimulation in the PAG or the NRM (Light, et al., 1986, but are generally excited by morphine injections into the NRM (LeBars, et al., 1980). Furthermore, intrathecal administration of morphine has been shown to selectively depresses some Lamina V neurons (Le Bars, Menetrey, Conseiller & Besson, 1975) suggesting that nociceptive information is inhibited directly and indirectly by descending pathways at the level of the dorsal horn.

D. Modulation of Primary Afferent Inputs in the Spinal Cord

1. History

Early recordings from dorsal horn neurons showed that afferent inputs at the first synapse in the pathway could be modulated under different circumstances. Differences between spinalized preparations and anesthetized preparations provided evidence for descending influences on neurons in the spinal cord (Wall, 1967). The Gate Control Theory (Melzack & Wall, 1965) was based on observations of presynaptic inhibition of A-fiber responses by C-fiber volleys and presynaptic facilitation of C-fiber inputs by A-fiber volleys (Eccles, Eccles & Magni, 1961; Howland, Lettvin, McCulloch, Pitts & Wall, 1965; Mendell & Wall, 1964; 1965). However, Price & Wagman (1970) showed that C-fiber activity was not necessary for A-fiber inhibition and A-fibers were not necesary for C-fiber facilitation, indicating that additional mechanisms were involved. Mounting evidence for descending modulation of nociceptive information in the spinal cord opposed many premises of the original Gate Control Theory (for review, see Nathan, 1976), but the basic premise that neurons in the dorsal horn modulate incoming afferent information has been supported by many groups (eg., Price, Hull & Buchwald, 1971; Handwerker, Iggo & Zimmerman, 1975; Cervero, Iggo & Molony, 1976; Repkin, Wolf & Anderson, 1976). In light of this more recent information, the theory of Melzack and Wall has been restated; the brain receives messages about pain through a gate-controlled system which can be influenced by injury signals, other types of afferent input, and descending control (Melzack & Wall, 1978). It is not clear how much of this modulation is due to the balance of primary afferent input and how much is due to descending inhibition of the brainstem because complex synaptic circuitry of the spinal cord has not been worked out.

2. Opioid Effects on Central Terminals of Primary Afferents

Modulation of primary afferent input is not restricted to activation of either A- or C-fibers. In addition, descending inhibitory pathways have been shown to have direct and indirect modulatory actions at the central terminal of primary afferents. Some evidence for these interactions have been demonstrated for GABA (eg., Curtis, Duggan, Felix & Johnston, 1971; Levy, Repken & Anderson, 1971; 1974; Barber, Vaughn, Saito, McLaughlin & Roberts, 1978), glutamate (Schneider & Perl, 1985), norepinephrine (Jones & Gebhart, 1986; Grudt & Williams, 1995), serotonin (Johannsson, et al., 1981; Travagli & Williams, 1995) and opioids (Duggan, Hall & Headley, 1976; 1977). The focus of this section will be on the effects of opioids in the dorsal horn of the spinal cord, and specifically on central terminals of primary afferents.

Intrathecal administration of morphine has been demonstrated to induce analgesia (Yaksh & Rudy, 1976), while intrathecal naloxone administered in the absence of morphine produces hyperalgesia (Woolf, 1980). These findings suggest a role for endogenous opioids in the spinal cord. In fact, enkephalin axonal endings make direct contacts with ascending dorsal horn projection neurons (Ruda, 1982) and are especially dense in laminae I and II (Glazer & Basbaum, 1981; Waksman, et al., 1986). There are also μ-, κ- and δ-receptor binding sites within the spinal cord (Ninkovic, Hunt & Gleave, 1982; Maekawa, et al., 1994), some of which are located presynaptically on primary afferent neurons. Both neonatal capsaicin treatment which lesions C-fibers and dorsal rhizotomy decrease opiate binding by 50% in the upper dorsal horn (LaMotte, Pert & Snyder, 1976; Gamse, Holzer & Lembeck, 1979; Fields, Emson, Leigh, Gilbert & Iverson, 1980).

Functionally, intrathecal administration of opioids have been shown to effect both dorsal horn neurons and primary afferents. Opioids have also been shown to inhibit increased C-fiber terminal excitability induced by A-fiber stimulation (Woolf & Fitzgerald, 1982) and noxious stimuli-induced neurotransmitter release into the spinal cord (MacDonald &

Nelson, 1978; Mudge, Leeman & Fischbach, 1979; Kangrga & Randic, 1991. More specifically, μ- and δ-agonists, but not the κ-selective agonist, dynorphin, inhibit noxious stimuli-induced release of substance P from the dorsal horn (Hirota, et al., 1985; Lembeck & Donnerer, 1985). Recordings from wide-dynamic range neurons in laminae IV - VI have determined that κ-agonists selectively inhibit noxious responses when applied directly to these neurons, but μ-agonists were more effective when microiontophoresed into the substantia gelatinosa (Duggan, et al., 1976; 1977; Johnson & Duggan, 1981; Fleetwood-Walker, Hope, Mitchell, El-Yassir & Molony, 1988). Therefore opioid receptors are probably preferentially located for different functions. μ-receptors are most likely associated with primary afferent terminals in the substantia gelatinosa (Johnson & Duggan, 1981; Maekawa, et al., 1994), but κ-receptors seem to be more effective in deeper laminae.

3. General Mechanisms of Opioid Action

Opioid activation of the opioid receptors (μ-, κ-, and δ-) has been shown to result in three classical actions: inhibition of adenylyl cyclase, hyperpolarization, and inhibition of neurotransmitter release (for review see Duggan & North, 1984; Grudt & Williams, 1995). Opioid inhibition of adenylyl cyclase has been studied in detail in cell lines and has become a model for tolerance and dependence associated with chronic administration of opioids (eg., Collier & Francis, 1975; Sharma & Klee, 1975; 1977; Law & Loh, 1982). Opioids have been shown to inhibit prostaglandin-induced increases in cAMP in brain homogenates (Collier & Roy, 1974) and neuroblastoma x glioma hybrid cells (Traber, Fischer, Latzin & Hamprecht, 1975), but until recently, no functional effect of opioid inhibition of adenylyl cyclase had been observed (see Manuscript 1).

It has also been demonstrated that opioids *activate* adenylyl cyclase via Gs in cell lines (Cruciani, Dvorkin, Morris, Crain & Makman, 1993) and Gi/Go in the olfactory bulb (Onali & Olianas, 1991; Olianas & Onali, 1993). Interestingly, it has been suggested that opioid activation of adenylyl cyclase in olfactory bulb may be the result of βγ subunit

interaction with adenylyl cyclase (Tang & Gilman, 1991). Another group has proposed dual effects of opioids in primary afferent neurons. In their studies, low concentrations of opioids increased cAMP levels and prolonged action potential durations through activation of Gs, but high concentrations inhibited adenylyl cyclase and decreased action potential durations via the usual pertussis toxin-sensitive Gi/Go pathway in dorsal root ganglion neurons and F11 cells (Higashi, Shinnick-Gallagher & Gallagher, 1982; Shen & Crain, 1989; Cruciani, Dvorkin, Morris, Crain & Makman, 1993). The functional significance of this dual modulation by opioids is not understood and requires further investigation.

Opioid-induced hyperpolarization occurs via Gi-mediated activation of inwardly rectifying K^+ channels by all three receptor subtypes (Williams, Egan & North, 1982; North, Willliams, Surprenant & Christie, 1987). Opioid-induced hyperpolarizations have been observed in many areas of the central nervous system, such as the substantia nigra (Lacey, Mercuri & North, 1989), ventral tegmental area (Johnson & North, 1992), substantia gelatinosa (Grudt & Williams, 1993; 1994), hippocampus (Madison & Nicoll, 1988), and the nucleus raphe magnus (Pan, Williams & Osborne, 1990). In contrast, it has been suggested that low concentrations of opioids decrease K^+ conductances by activation of Gs in both F11 cells and cultured primary afferent neurons (Fan, Shen & Crain, 1991; 1993). However, other groups looking for opioid effects on membrane properties of primary afferent neurons have not made similar observations (Shefner, North & Zukin, 1981; Williams & Zieglgansberger, 1981).

Opioids have also been shown to inhibit voltage-gated Ca^{+2} currents in primary afferent neurons (Gross & MacDonald, 1987; Schroeder, Fischbach, Zheng & McCleskey, 1991) and to inhibit a Ca^{+2} -sensitive component of action potentials (Higashi, et al., 1982; Werz & MacDonald, 1982; Shen & Crain, 1989). Although the latter action was originally attributed to the decrease of a potassium current (Werz & MacDonald, 1983), it was later shown that opioids have no inhibitory effect on potassium channels when calcium channels

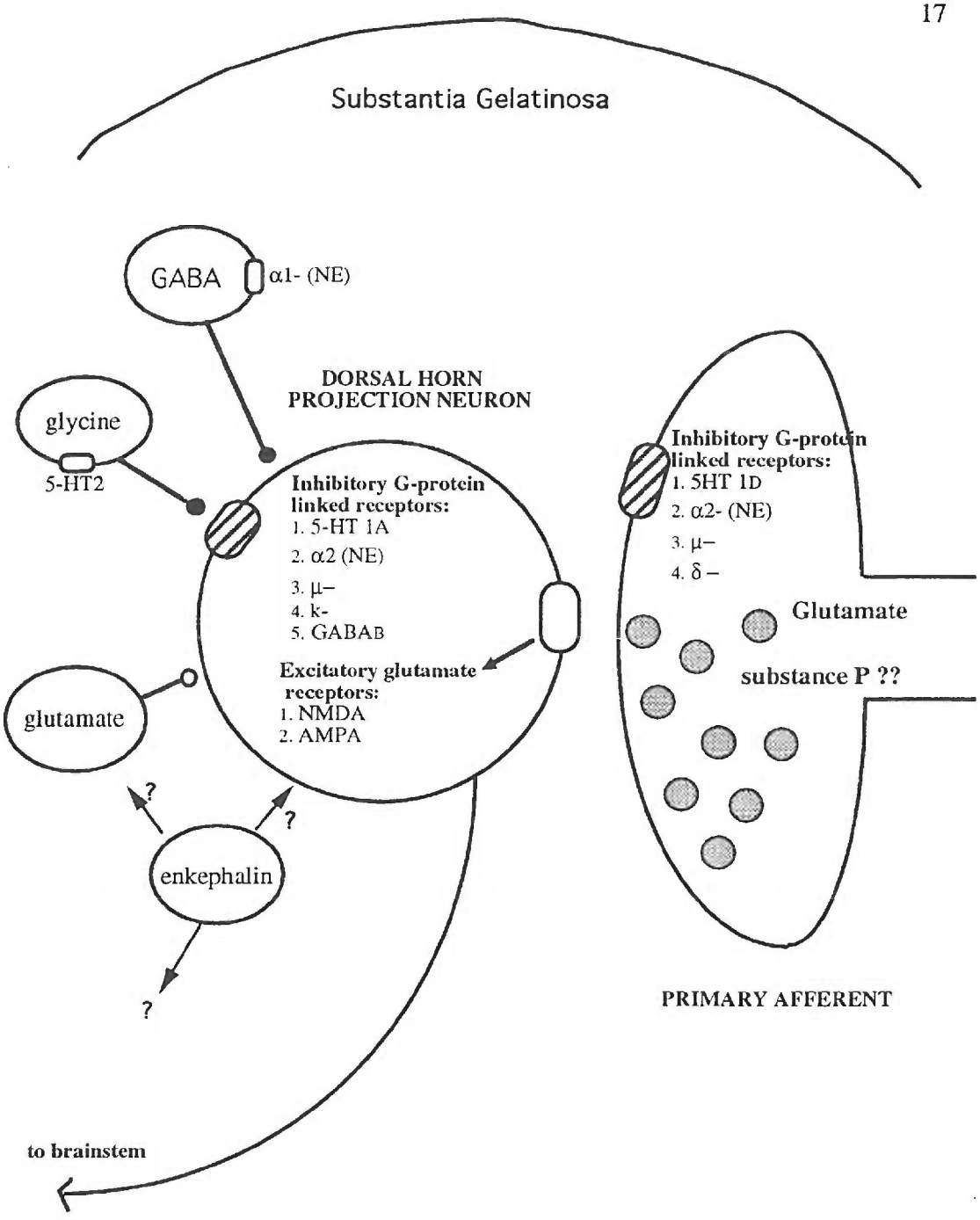

Figure 2. Modulation of Pain at the Central Terminal of the Primary Afferent.

are blocked in these neurons (Atkins & McCleskey, 1993).

Opioids inhibit neurotransmitter release from primary afferent terminals in the spinal cord (MacDonald & Nelson, 1978; Mudge, Leeman & Fischbach, 1979) and also inhibit IPSPs and EPSPs on central neurons (eg., Nicoll, Alger & Jahr, 1980; Chieng & Christie, 1994; Grudt & Williams, 1994). These effects may result from either inhibition of Ca^{+2} channels (eg., Gross & MacDonald, 1987; Schroeder, Fischbach, Zheng & McCleskey, 1991; Hori, Endo & Takahashi, 1992), hyperpolarization (for review, North and Williams, 1983), or a direct effect on the neurotransmitter release mechanism as suggested by Capogna, Gahwiler & Thompson (1993).

It is clear that nociceptive information can be mediated at all levels of the pain pathway. Although the responses of spinal cord neurons have been characterized in many cells, the synaptic circuitry within the cord, role of neurotransmitters and neuropeptides, and integration of impulses from the periphery are still not understood. To this point, the focus of the Introduction has been on the historical basis for our current concepts and beliefs about pain. To reiterate, many of the basic concepts in pain were proposed in the early 1900's. Since then, evolution of techniques (immunochemistry, electrophysiology, etc.) has allowed detailed characterization of the components of the pain system and the interactions at all levels of the nervous system. Consequently, the system has been revealed to be much more complex than could have been hypothesized at the turn of the century. However, many of these early ideas are still the basis of our current thoughts about pain and many basic mechanistic questions have not been answered.

II. REGULATION OF PAIN AT THE PERIPHERAL TERMINALS OF PRIMARY AFFERENTS

A. Peripheral Pain

Tissue damage elicits both acute and chronic pain. Acute pain can be attributed to the initial excitation of nociceptors (Adrian, 1927; Sinclair & Stokes, 1964; Bessou & Perl, 1969).

Both the initial excitation and tissue damage contribute to the inflammatory response generally associated with the production of hyperalgesia and allodynia. Hyperalgesia is a state of decreased nociceptor thresholds in the area of injury and is primarily due to sensitization of polymodal nociceptors (Lynn, 1977; Fitzgerald, 1979). Allodynia is a phenomenon where the tissue surrounding the damaged area is sensitized and innocuous stimuli are sufficient to cause intense pain. Allodynia (also called secondary hyperalgesia) can be initiated by antidromic stimulation of peripheral nerve fibers and blocked with local anesthetic in the area of injury (Fitzgerald, 1979) suggesting that it is due to release of chemicals into the periphery. Both hyperalgesia and allodynia occur simultaneously with the inflammatory response and are thought to be the result of changes in the area of tissue damage induced by the inflammatory response. Inflammation induces resting discharge and lower thresholds of activation (sensitization) in small diameter primary afferents (Coggeshall, Hong, Langford, Schaible & Schmidt, 1983; Schaible & Schmidt, 1985.

Acute excitation of nerve fibers by tissue damage begins a cascade of events in the peripheral tissue (see Figure 3). The axon-reflex release of neuropeptides (substance P, calcitonin gene-related peptide, etc.) from the peripheral end of primary afferents and release of substances from mast cells and immunocytes invading the damaged tissue probably begin the inflammatory response. Perfusates collected after tissue damage and inflammation cause a burning pain and flare and sensitize nociceptors (Armstrong, Jepson, Keele & Stewart, 1957; Chapman, Ramos, Goodell & Wolff, 1961; Perl, 1976) when injected into normal skin. Some of the substances that have been isolated from inflammatory exudates and studied in detail include bradykinin, substance P, serotonin, norepinephrine, and prostaglandin E2. The exact order of events in the inflammatory cascade is not known, but the release and/or production of all of these substances have been implicated in the production and maintenance of both hyperalgesia and inflammation.

The mechanisms by which substances released from inflamed tissues induce hyperalgesia are both direct and indirect (see Figure 4). Direct excitation of nociceptors has been shown for bradykinin (eg., Beck & Handwerker, 1974), serotonin (Neto, 1978; Taiwo & Levine,

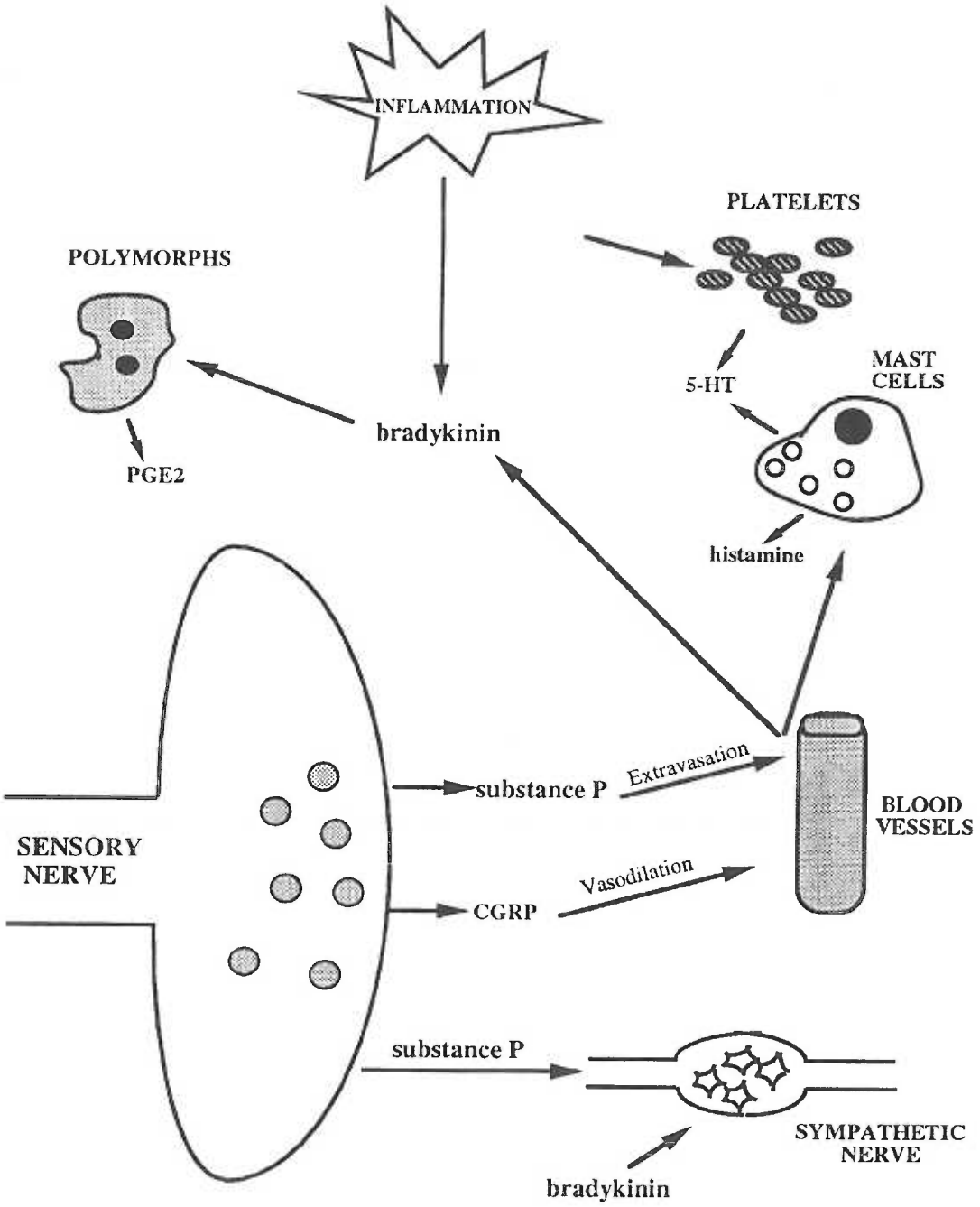

Figure 3. Neuropeptides released from peripheral terminals of primary afferents have pleiotropic effects. (Adapted from Dray and Bevan, 1993).

TISSUE DAMAGE

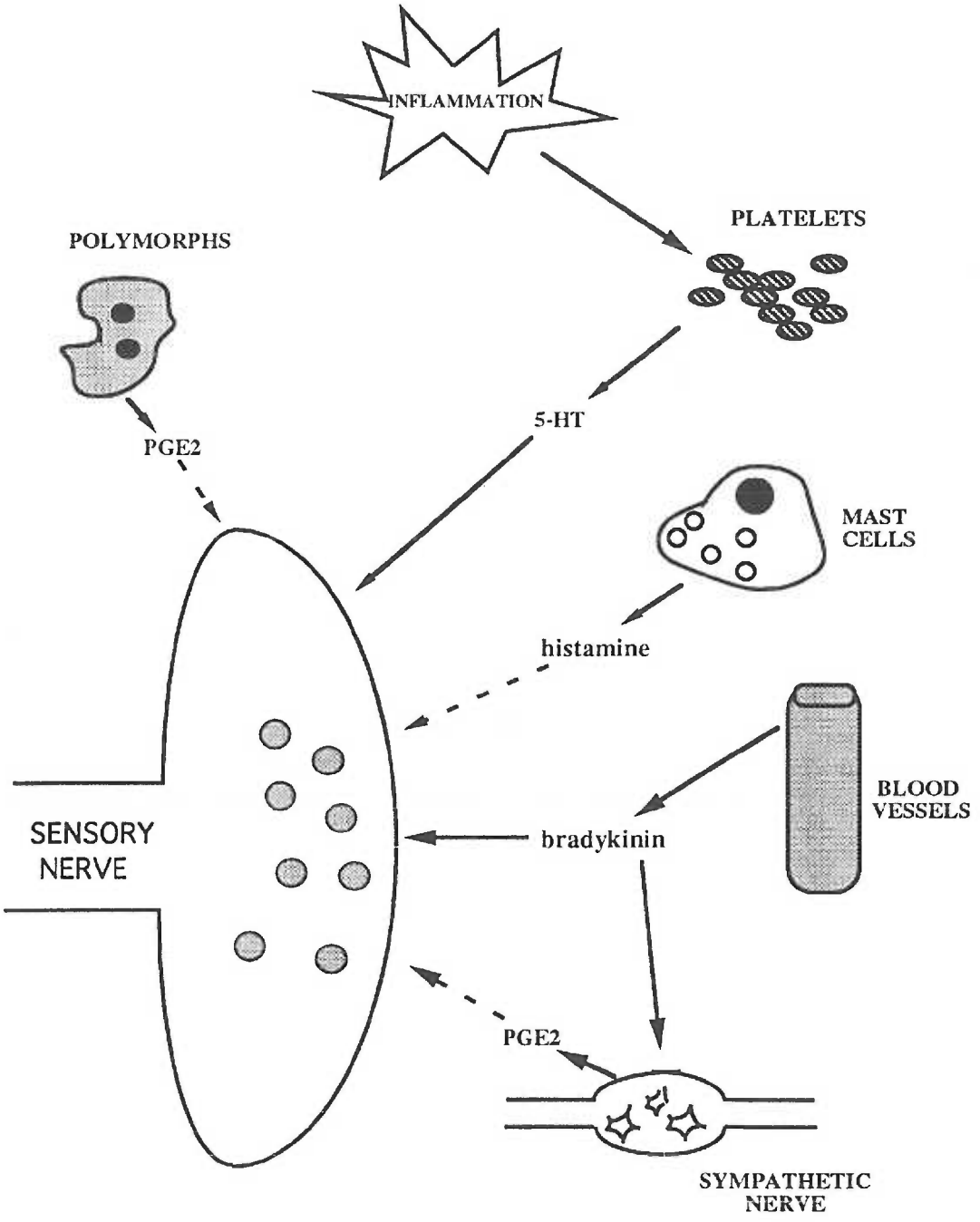

Figure 4. Substances released during the inflammatory response can either sensitize (dashed lines) or directly excite (continuous lines) peripheral endings of primary afferents. (Adapted from Dray and Bevan, 1993).

1992), norepinephrine (Sato, Suzuki, Iseki & Kumazawa, 1993), substance P (Randic & Miletic, 1977; Stanfield, Nakajima & Yamaguchi, 1985) and prostaglandin E2 (eg., Handwerker, 1976; Schaible & Schmidt, 1988). Indirect effects of these substances include sensitization of nociceptors, increased neurotransmitter release, increased sympathetic activity, vasodilation of blood vessels and mast cell degranulation. Production and release of substances associated with indirect actions of inflammatory exudates results in secondary excitation of nociceptors which continues the inflammatory cycle. It is not surprising that many of these substances have been shown to work in concert (Kessler, Kirchoff, Reeh & Handwerker, 1992). Therefore, none of the pain phenomena studied (i.e. hyperalgesia, inflammation, vascular permeability, primary afferent sensitization, etc.) exist in isolation, making interpretations of data extremely difficult. It is not known how all of these events occur temporally in relation to one another or the extent to which each phenomenon contributes to our perception of pain.

1. Sensitization of Nociceptors

Lewis (1936) determined that hyperalgesia was the result of peripheral sensitization of nociceptors. He noted that hyperalgesia could be elicited by electrical stimulation of cutaneous nerve fibers or strong mechanical pressure to the skin, but the production of hyperalgesia was blocked if he anesthetized either the nerve or the area where the mechanical pressure was applied. Thus, he proposed the 'nocifensor hypothesis' that stated the afferent nerves themselves were sufficient to produce hyperalgesia through antidromic activity and release of a pain-producing agent in the periphery. Sensitization of nociceptors is a specialized phenomenon in that nociceptors have a period of prolonged excitation and decreased thresholds after repeated or prolonged stimulation (Perl, 1976). In contrast, low-threshold receptors typically fatigue upon repeated stimulation. Repeated stimulation of peripheral nerves has been shown to induce sensitization in C- and A-fiber polymodal nociceptors (Campbell, Meyer & LaMotte, 1979; Fitzgerald, 1979; Meyer & Campbell, 1981; LaMotte, Thalhammer, Torebjork & Robinson, 1982). However, only C-fiber sensitization has been correlated with pain sensations in human psychophysical experiments, and hyperalgesia was not affected by A-fiber conduction block (LaMotte,

Thalhammer, Torebjork & Robinson, 1982; 1983; Shir & Seltzer, 1990). Shir & Seltzer (1990) confirmed that C-fiber sensitization was important for the production of hyperalgesia by treating neonatal rats with capsaicin and observing that they could not produce thermal hyperalgesia in the sciatic nerves of these animals. Allodynia, on the other hand, was still present, indicating a role of A-fiber sensitization in allodynia. In addition, it has been proposed that sensitization of different fibers in different tissues may be important for hyperalgesia (Campbell & Meyer, 1983). A-fiber sensitization may play a role in the glabrous skin of the hand, while C-fiber sensitization is necessary for hairy skin of the hand (Campbell, Meyer & LaMotte, 1979; Meyer & Campbell, 1981). While sensitization of nociceptors is closely associated with hyperalgesia and allodynia, the mechanisms of nociceptor sensitization are not known. It is thought that interactions of primary afferents and substances released and/or produced by the inflammatory cascade are responsible. Serotonin (Sicuteri, Fanciullacci, Franchi & Del Bianco, 1964), PGE2 (Mense, 1981; Pitchford & Levine, 1991; Birrell, McQueen, Iggo & Grubb, 1993) and bradykinin (Birrell, McQueen, Iggo & Grubb, 1993; Rueff & Dray, 1993) have all been shown to sensitize nociceptive primary afferents.

2. Increased Release of Neurotransmitters

Primary and secondary excitation of nociceptors after tissue damage can increase the release of neuropeptides and neurotransmitters into the spinal cord and the periphery (Andreeva & Rang, 1993). Antidromic activity does not seem to be sufficient to alter the sensitivity of nociceptors (Reeh, Kocher & Jung, 1986) and indicates that release of neuropeptides into the periphery may not be sufficient to produce hyperalgesia. However, several factors are simultaneously released with antidromic stimulation (Chahl & Ladd, 1976). Peripheral release of substance P and CGRP has been shown to have many actions in the periphery, such as vasodilation of blood vessels, production of prostaglandins from inflammatory cells and release of histamine via degranulation of mast cells (for review, see Otsuka & Yoshioka, 1993). These substances (bradykinin, serotonin, histamine and substance P) in turn excite nociceptors and sympathetic efferents (Saria, et al., 1988; Andreeva & Rang, 1993; Birrell, McQueen, Iggo & Grubb, 1993; Green, Luo, Heller & Levine, 1993)

resulting in continued activation of the inflammatory cascade.

3. Vasodilation and Interactions with the Sympathetic Nervous System

Lewis (1936) proposed in the 'nocifensor hypothesis' that the afferent nerves themselves were sufficient to produce hyperalgesia through antidromic activity and release of a pain-producing agent in the periphery that could produce vasodilation. These fibers were determined to be the afferent fibers of peripheral nerves (Bayliss, 1901). Antidromic stimulation elicits capsaicin-sensitive cutaneous vasodilation, supporting the idea of a dual sensory-efferent function of capsaicin-sensitive nociceptors (Langley, 1923; Janscó, Janscó-Gábor & Szolcsányi, 1967; Kenins, 1980; Szolcsányi, 1988). Antidromic stimulation-produced vasodilation and plasma extravasation are dependent on the sympathetic nervous system (Engel, 1941; Levine, Taiwo, Collins & Tam, 1986; Coderre, Basbaum & Levine, 1989). Sympathetic fibers are closely associated with blood vessels surrounding peripheral sensory ganglia (Owman & Santini, 1966; Stevens, Hodge & Apkarian, 1983) and afferent fibers (Roberts & Levitt, 1982; Stevens, Hodge & Apkarian, 1983; Quigg, Elfvin & Aldskogius, 1990). Sympathetic efferents have been shown to sprout in the event of nerve injury to form basket-like structures around injured nerves (McLachlan, Jänig, Devor & Michaelis, 1993) suggesting that release of norepinephrine from sympathetic terminals may act on both blood vessels and primary afferents.

Hyperalgesia can be blocked by α_2-antagonists and sympathectomies (Levine, et al., 1986; Sato, Suzuki, Iseki & Kumazawa, 1993) indicating that the sympathetic system has a role in hyperalgesia. A-fiber nociceptors can be excited by sympathetic stimulation, but there is no evidence for activation of C-nociceptors in normal tissue (Roberts & Levitt, 1982; Shea & Perl, 1985). However, sympathetic stimulation excites both Aδ- and C-fibers in injured nerve preparations (Devor & Jänig, 1981; Blumberg & Jänig, 1984; Sato & Perl, 1991; Devor, Jänig & Michaelis, 1994), and sympathectomies have been shown to block hyperalgesia and norepinephrine-induced potentiation of hyperalgesia (Levine, et al., 1986).

The role of the sympathetic nervous system in neurogenic-induced inflammation is complex. Neurogenic vascular permeability is dependent on sympathetic terminals (Coderre, Basbaum & Levine, 1989), but exogenous norepinephrine actually decreases plasma extravasation associated with inflammation (Green, 1974; Green, Luo, Heller & Levine, 1993). The role of plasma extravasation has been hypothesized to be involved in repairing the tissue damage as increased plasma extravasation is associated with decreased injury (Coderre, Chan, Helms, Basbaum & Levine, 1991). Therefore, the release of norepinephrine from sympathetic terminals must act indirectly to produce a substance that increases plasma extravasation. The production of prostaglandin E2 (PGE2) has been proposed as a likely candidate. Intradermal arachidonic acid (the precursor to prostaglandins) produces hyperalgesia that is blocked by indomethacin and sympathectomies (Gonzales, Goldyne, Taiwo & Levine, 1989). Indomethacin is an inhibitor of cyclo-oxygenase, an enzyme that synthesizes prostaglandins from arachidonic acid, so these experiments suggest that sympathetic terminals are the source of prostaglandins (Levine, et al., 1986). Bradykinin-induced release of norepinephrine (Green, et al., 1993) and PGE2 production (Gonzales, et al., 1989) was much decreased in sympathectomized animals. Interestingly, prostaglandins seem to have a feedback inhibition of neurotransmitter release in sympathetic ganglia (Dun, 1980; Belluzi, et al., 1982).

B. PGE2 and Peripheral Pain

PGE2 has been correlated with and is released during many pain states, such as burn (Arturson, Hamberg & Jonsson, 1973), blisters (Goldyne, Winkelmann & Ryan, 1973), and inflammation (Barbieri, Orzechowski & Rossi, 1977). PGE2 has been shown to produce hyperalgesia by direct excitatory effects on primary afferent terminals, as well as indirect effects associated with the inflammatory response (for review, see Andreeva & Rang, 1993). Repeated injections of PGE2 into the skin induces both a swelling of the area of injection and a sustained hyperalgesia (Willis & Cornelsen, 1973; Khasar, Green & Levine, 1993). PGE2-induced hyperalgesia appears to be a direct action on peripheral

terminals as hyperalgesia is not affected by blockade of indirect mechanisms; 6-OHDA depletion of sympathetic terminals or compound 48/80 blockade of mast cell degranulation (Taiwo & Levine, 1989a; Taiwo, Bjerknes, Goetzl & Levine, 1989).

PGE2 can also contribute indirectly to hyperalgesia and the inflammatory response through its actions on release of neurotransmitters, plasma extravasation, and increasing primary afferent excitability. PGE2 has been shown to increase the release of neuropeptides (substance P, CGRP, etc.) and enhance bradykinin-stimulated release from primary afferent neurons (Nicol, Klingberg & Vasko, 1992; Vasko, Campbell & Waite, 1994; Hingtgen, Waite & Vasko, 1995). Indomethacin blocks these effects (Vasko, Campbell & Waite, 1994). It has also been demonstrated that PGE2 can cause an increase in local vascular dilation (Williams & Peck, 1977) that enhances vascular permeability induced by bradykinin, histamine and serotonin (Crunkhorn & Willis, 1971; Williams & Morley, 1973; Green, et al., 1994). The increase in permeability during experimentally-induced inflammation can be inhibited by indomethacin (Moncada, Ferreira & Vane, 1973).

Bradykinin, serotonin and histamine have all been shown to activate primary afferent neurons. Bradykinin production is increased in tissue damaged areas and has both direct and indirect effects on inflammatory processes (for review, see Dray & Perkins, 1993). Bradykinin hyperalgesia and plasma extravasation are thought to be due to bradykinin-induced production of prostaglandins (Lembeck, Popper & Juan, 1976; Dray, Patel, Perkins & Rueff, 1992). Although algogenic substances such as bradykinin and serotonin increase the excitability of all types of primary afferent fibers (Beck & Handwerker, 1974), PGE2 seems to specifically sensitize polymodal nociceptors with small-diameter myelinated and unmyelinated fibers (eg., Handwerker, 1976; Mense, 1981; Martin, Basbaum, Kwiat, Goetzl & Levine, 1987; Schaible & Schmidt, 1988; Rueff & Dray, 1993). These results support the hypothesis that polymodal nociceptors (both Aδ- and C) play a major role in inflammatory pain. Inflammation in the knee joint produces a resting discharge not normally seen in fine articular afferents and causes a larger response with higher frequency discharge to passive movements than in normal states (Coggeshall, Hong, Langford,

Schaible & Schmidt, 1983; Schaible & Schmidt, 1985). These effects can be mimicked by close intra-arterial injection of prostaglandins (Schepelmann, Meßlinger, Schaible & Schmidt, 1992) and blocked by indomethacin suggesting that PGE2 causes increased primary afferent firing during inflammation (Heppelmann, Pfeffer, Schaible & Schmidt, 1986). PGE2 also has an excitatory effect on small-diameter primary afferents on its own (Heppelmann, Schaible & Schmidt, 1985; Yanagisawa, Otsuka & Garciá-Arrarás, 1986; Schaible & Schmidt, 1988), but this effect may be selective for chemosensitive fibers (Birrell & McQueen, 1993). In other experiments, PGE2 has no effect when administered alone (Pitchford & Levine, 1991; Nicol & Cui, 1994), but is part of the 'inflammatory soup' needed to mimic excitatory effects of inflammation (Kessler, Kirchoff, Reeh & Handwerker, 1992).

The mechanism of action of prostaglandins in producing hyperalgesic effects is unknown, but it is likely that prostaglandin stimulation of cAMP is involved. Hyperalgesia is associated with activation of Gs (Taiwo & Levine, 1989) and an increase in cAMP levels (Taiwo, et al., 1989; Pitchford & Levine, 1991; Taiwo & Levine, 1991; Taiwo, Heller & Levine, 1992; Rueff & Dray, 1993). PGE2 can increase the levels of cAMP in the brains of mice (Wellman & Schwabe, 1973) and can facilitate peptide release and increase hyperalgesia through a cAMP-dependent mechanism (Pitchford & Levine, 1991; Khasar, Ho, Green & Levine, 1994; Hingtgen, et al., 1995). PGE2 can also increase calcium conductances (Nicol, et al., 1992), and it has been suggested that PGE2-induced hyperalgesia is both a cAMP and Ca^{+2}-dependent process (Ferreira & Nakamura, 1979). The inhibitor peptide of protein kinase A has been shown to inhibit PGE2 sensitization of bradykinin excitation in capsaicin-sensitive dorsal root ganglion neurons (Cui & Nicol, 1995) indicating that protein kinase A may play a role in increased primary afferent excitability (Ouseph, Khasar & Levine, 1995).

C. Peripheral Actions of Opioids

1. Effects of Opioids on Primary Afferent Somata

Opioid actions have been shown to decrease neurotransmitter release and modulate primary afferent activity in the spinal cord (see above). In addition, it has been demonstrated that opioid receptors are transported bidirectionally (Zarbin, Wamsley & Kuhar, 1990), and they are localized to capsaicin-sensitive fibers (Laduron, 1984). However, opioid modulation of membrane properties of primary afferents has not been demonstrated (Shefner, North & Zukin, 1981; Williams & Zieglgansberger, 1981), and direct application of morphine onto nerve fibers also failed to change heat-evoked activity of C-fibers (Senami, et al., 1986). Opioids do inhibit Ca^{+2} and Ca^{+2}-sensitive K^{+} channels in cultured primary afferents (Gross & Macdonald, 1987; Schroeder, et al., 1991; Atkins & McCleskey, 1993), and this may result in decreased neurotransmitter release (Mudge, et al., 1979; Macdonald & Nelson, 1978). Although opioids do not have a huge effect on excitability of primary afferent somata in control conditions, axonal transport of opioid receptors is enhanced to the periphery during inflammation (Hassan, Ableitner, Stein & Herz, 1993), and peripheral injections of opioids have been shown to be very effective inhibitors of peripheral hyperalgesia and inflammatory pain.

2. Opioid Actions at the Peripheral Terminals of Primary Afferents

Opioids have been shown to inhibit all of the changes that occur during inflammation. Activation of peripheral opioid receptors (μ-, δ-, and κ-) inhibits inflammatory-induced sensitization and hyperalgesia (Stein, Millan, Shippenberg & Herz, 1988; Stein, Millan, Shippenberg, Peter & Herz, 1989), as well as increased nerve fiber activity in inflamed tissue (Russell, Schaible & Schmidt, 1987; Andreev, Urban & Dray, 1994). Intra-arterial injections of μ- and δ-agonists have been shown to inhibit the release of substance P into the knee joint cavity after either antidromic stimulation or intradermal injections of capsaicin (Yaksh, 1988). These results indicate that opioids may be very effective in the inhibition of inflammatory pain. Opioids also decrease neurogenic and bradykinin-induced plasma extravasation (Barthó & Szolcsányi, 1981; Green & Levine, 1992), as well as

hyperalgesia that is dependent on sympathetic ganglia (Taiwo & Levine, 1991b; Green & Levine, 1992). Thus, the peripheral actions of opioids may be selective for pain-altered states, and specifically, inflammatory pain.

Local injections of opioids acting on peripheral receptors have been shown to inhibit pain, but the origin of endogenous opioid ligands for these peripheral receptors is not known. Increased excitability of nociceptive afferents from acutely inflamed tissue was not altered by injections of naloxone suggesting that there was no endogeneous opioid inhibition during inflammation (Schepelmann, Meßlinger, Schaible & Schmidt, 1995). However, endogenous peripheral opioids have been shown to cause antinociception in an inflamed paw during the cold water swim stress test (CWSS) (Parsons & Herz, 1990). This antinociception is blocked by intraplantar injection of naloxone, but not iintravenous or subcutaneous injections, confirming a peripheral release of opioids during the stress response (Stein, Gramsch & Herz, 1990). Therefore, it is likely that yet another system is involved in the inflammatory response and pain perception.

On the basis of the preceding studies, it was proposed that β-endorphin release into the bloodstream from the adrenal gland was the source of endogenous peripheral opioids. However, although β-endorphin antibodies attenuated the CWSS test (Stein, et al., 1990), adrenalectomy had no effect on the induction of analgesia and hypophysectomy had very little effect (Parsons, Czlonkowski & Stein, 1990). On the other hand, immunosuppressants have been shown to block CWSS - induced antinociception, and cytokines (eg., tumor necrosis factor α and interleukin-6) increased nociceptive thresholds in a naloxone-sensitive manner (Czlonkowski, Stein & Herz, 1993). The results from the CWSS test (Parsons & Herz, 1990) and immunocytochemical evidence that both β-endorphin and met-enkephalin are increased in immunocytes localized to inflamed tissue support a hypothesis that immune cells contain and release opioids into the periphery (Stein, et al., 1990). Thus, the immune system probably plays an integral role in opioid

suppression of inflammation and inflammatory pain. The evidence on this subject is quite recent, so many more experiments are needed to assess the significance of this interaction to pain perception.

The mechanism of opioid inhibition of peripheral pain is not known. As stated earlier, opioids have no effect on membrane properties of primary afferent neurons, but have been shown to inhibit Ca^{+2} currents, as well as forskolin and PGE2-stimulated adenylyl cyclase activity (Traber, Fischer, Latzin & Hamprecht, 1975; Brandt, et al., 1976; Makman, Dvorkin & Crain, 1988). PGE2-induced hyperalgesia is inhibited by both μ-agonists and adenylyl cyclase inhibitors in inflamed tissue (Ferreira & Nakamura, 1979; 1982; Stein, Millan & Shippenberg, 1988; Levine & Taiwo, 1989). Similarly, intrathecal μ- and δ-agonists inhibit basal and forskolin-stimulated cAMP levels in the spinal cord in monoarthritic rats (Przewlocka, Lason & Przewlocki, 1991). Therefore, opioid inhibition of adenylyl cyclase may play a major role in alleviating peripheral pain. The mechanisms by which increased levels of cAMP in primary afferents contribute to inflamation and hyperalgesia are not known.

D. Summary

Stimulation of primary afferents by tissue damage can release neurotransmitters and neuropeptides into both the spinal cord and periphery. The release of neuropeptides from primary afferents, as well as from damaged tissue, can cause vasodilation of blood vessels, increased excitability of primary afferent and sympathetic efferent fibers, and continued release of substances implicated in the inflammatory response. The order of these responses is not understood, but it is clear that all of these systems work in concert to produce pain and repair tissue damage.

III. GENERAL AIMS

The aims of this thesis were to determine a potential mechanism by which inflammatory substances transduce a chemical signal into increased electrical activity in nociceptive primary afferents. In order to focus our research, we chose to study the effects of PGE2 on the non-selective cation current, I_h. I_h is a hyperpolarization-activated inward current with an activation range near the resting membrane potential of primary afferent neurons. Modulation of I_h by increases in cAMP concentration has been shown to increase the firing rate of cardiac myocytes (DiFrancesco, Ducouret & Robinson, 1989; Denyer & Brown, 1990) and to change the firing patterns of central neurons (McCormick & Pape, 1990). Although I_h has already been described in primary afferent neurons, the modulation by of I_h by cAMP has not been studied. PGE2-induced hyperalgesia is associated with increased levels of cAMP, and opioids are known to inhibit adenylyl cyclase, therefore the first section of the thesis is focused on the possible modulation of I_h by PGE2 and opioids.

The majority of the experiments in this thesis involved whole-cell recordings from cultured nodose ganglion neurons. The nodose ganglion contains cell bodies of afferent fibers from visceral organs, such as the esophagus, trachea, heart, lungs and stomach (Chase & Ranson, 1914). The main projection of these neurons is to the nucleus tractus solitarius (NTS) of the brainstem (Kalia & Mesulam, 1980; 1982). These fibers have conduction velocities in the A- and C-fiber range, but are predominately unmyelinated C-fibers (Agostoni, Chinnock, De Burgh-Daly & Murray, 1957). Nodose ganglion neurons are associated with substance P and calcitonin gene-related peptide, similar to small-diameter dorsal root ganglion neurons (MacLean, Bennett, Morris & Wheeler, 1989). The passive and active membrane properties of these cells in slice preparations and in whole ganglion are also A- and C-like (Gallego & Ezyaguirre, 1978; Stansfield & Wallis, 1985). Similar to spinal afferents, nodose afferents have been shown to express and transport opioid receptors to both terminals (Laduron, 1984), and these receptors are decreased in the NTS after vagotomy (Atweh and Kuhar, 1977). Approximately 75% of the opioid receptors are μ-receptors, although μ-, δ- and κ- receptors are localized to nodose ganglion neurons

(Zarbin, Wamsley & Kuhar, 1990). Interestingly, opioids have no effect on electrical membrane properties of these neurons when at rest (Shefner, et al., 1981). However, κ-receptors inhibit stimulated N-type calcium currents (Gross, Moises, Uhler & Macdonald, 1990). Although the responses of nodose afferents to noxious stimuli have been difficult to assess *in vivo*, they have similar characteristics to small-diameter primary afferents (Aδ- and C-) in the dorsal root ganglion and were chosen in this study because a large percentage of cells in the ganglion respond to opioids.

The second portion of this thesis deals with the mechanism of modulation of I_h by PGE2 and opioids. PGE2-induced hyperalgesia and sensitization of primary afferents was shown to be sensitive to the inhibitor peptide of protein kinase A (Cui & Nicol, 1995; Ouseph, 1995). Several different mechanisms of I_h modulation have been proposed in cardiac myocytes; phosphorylation by protein kinase A (Chang, Cohen, DiFrancesco, Rosen & Tromba, 1991), direct stimulation by G proteins (Yatani, Okabe, Codina, Birnbaumer & Brown, 1990), and direct stimulation by cAMP (DiFrancesco & Tortora, 1991). We were interested in determining the mechanism of cAMP augmentation of I_h in primary afferents.

REFERENCES

Adams, J. E. (1976). Naloxone reversal of analgesia produced by brain stimulation in the human. *Pain* **2**, 161-166.

Adrian, E. D. (1927). The impulses produced by sensory nerve endings. Part 4. Impulses from pain receptors. *Journal of Physiology* **62**, 33-51.

Adrian, E. D. (1931). The messages in sensory nerve fibers and their interpretation. *Proceedings of the Royal Society B* **109**, 1-18.

Adrian, E. D., Cattell, M. & Hoagland, H. (1931). Sensory discharges in single cutaneous nerve fibers. *Journal of Physiology* **72**, 377-391.

Agostoni, E., Chinnock, J. E., De Burgh Daly, M. & Murray, J. G. (1957). Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. *Journal of Physiology* **135**, 182-205.

Aimone, L.D. & Gebhart, G.F. (1987). Spinal monoamine mediation of stumulation-produced antinociception from the lateral hypothalamus. *Brain Research* **403**, 290-300.

Aimone, L. D., Jones, S. L. & Gebhart, G. F. (1987). Stimulation-produced descending inhibition from the periaqueductal gray and nucleus raphe magnus in the rat: mediation by spinal monoamines but not opioids. *Pain* **31**, 123-136.

Akil, H., Mayer, D. J. & Liebeskind, J. C. (1976). Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science* **191**, 961-962.

Ammons, W. S., Blair, R. W. & Foreman, R. D. (1983). Vagal afferent inhibition of primate thoracic spinaothalamic neurons. *Journal of Neurophysiology* **50**, 926-940.

Andreev, N., Urban, L. & Dray, A. (1994). Opioids suppress spontaneous activity of polymodal nociceptors in rat paw skin induced by ultraviolet irradiation. *Neuroscience* **58**, 793-798.

Andreeva, L. & Rang, H. P. (1993). Effect of bradykinin and prostaglandins on the release of calcitonin gene-related peptide-like immunoreactivity from the rat spinal cord *in vitro*. *British Journal of Pharmacology* **108**, 185-190.

Armstrong, D., Jepson, J. B., Keele, C. A. & Stewart, J. W. (1957). Pain-producing substance in human inflammatory exudates and plasma. *Journal of Physiology* **135**, 350-370.

Arturson, G., Hamberg, M. & Jonsson, C. -E. (1973). Prostaglandins in human burn blister fluid. *Acta Physiologica Scandanavia* **87**, 270-276.

Atkins, P. T. & McCleskey, E. W. (1993). Characterization of potassium currents in adult rat sensory neurons and modulation by opioids and cyclic AMP. *Neuroscience* **56**, 759-769.

Atweh, S. F. & Kuhar, M. J. (1977). Autoradiographic localization of opiate receptors in rat brain. I. Spinal cord and lower medulla. *Brain Research* **124**, 53-67.

Atweh, S. F. & Kuhar, M. J. (1977b). Autoradiographic localization of opiate receptors in rat brain. II. The brainstem. *Brain Research* **129**, 1-12.

Atweh, S. F., Murrin, L. C. & Kuhar, M. J. (1978). Presynaptic localization of opiate receptors in the vagal and accessory optic systems: an autoradiographic study. *Neuropharmacology* **17**, 65-71.

Baccaglini, P. I. & Hogan, P. G. (1983). Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proceedings of the National Academy of Sciences USA* **80**, 594-598.

Balagura, S. & Ralph, T. (1973). The analgesic effect of electrical stimulation of the diencephalon and mesencephalon. *Brain Research* **60**, 369-379.

Barber, R. P., Vaughn, J. E., Saito, K., McLaughlin, B. J., & Roberts, E. (1978). GABAergic terminals are presynaptic to primary afferent terminals in the substantia gelatinosa of the rat spinal cord. *Brain Research* **141**, 35-55.

Barber, R. P., Vaughn, J. E., Slemmon, J. R., Salvaterra, P. M., Roberts, E. & Leeman, S. E. (1979). The origin, distribution and synaptic relationships of substance P axons in rat spinal cord. *Journal of Comparative Neurology* **184**, 331-352.

Barbieri, E. J., Orzechowski, R. F. & Rossi, G. V. (1977). Measurement of prostaglandin E2 in an inflammatory exudate: effects of nonsteroidal anti-inflammatory agents. *Journal of Pharmacology and Experimental Therapeutics* **201**, 769-777.

Barthó, L. & Szolcsányi, J. (1981). Opiate agonists inhibit neurogenic plasma extravasation in the rat. *European Journal of Pharmacology* **73**, 101-104.

Basbaum, A. I. & Fields, H. L. (1978). Endogenous pain control mechanisms: review and hypothesis. *Annals of Neurology* **4**, 451-462.

Basbaum, A. I. & Fields, H. L. (1984). Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annual Review of Neuroscience* **7**, 309-338.

Bayliss, W. M. (1901). On the origin from the spinal cord of the vaso-dilator fibres of the hind-limb, and on the nature of these fibres. *Journal of Physiology* **26**, 173-209.

Beart, P.M., Summers, R.J., Stephenson, J.A., Cook, C.J. & Christie, M.J. (1990). Excitatory amino acid projections ot the periaqueductal gray in the rat: a retrograde transport study utilizing D[^{3}H]Aspartate and [^{3}H]GABA. *Neuroscience* **34**, 163-176.

Beck, P. W. & Handwerker, H. O. (1974). Bradykinin and serotonin effects on various types of cutaneous nerve fibres. *Pflugers Archives* **347**, 209-222.

Behbehani, M. M. & Fields, H. L. (1979). Evidence that an excitatory connection between the periaqueductal gray and nucleus raphe magnus mediates stimulation-produced analgesia. *Brain Research* **170**, 85-93.

Behbehani, M. M., Jiang, M. & Chandler, S. D. (1990). The effect of [met]enkephalin on the periaqueductal gray neurons of the rat: an *in vitro* study. *Neuroscience* **38**, 373-380.

Belluzzi, O., Biondi, C., Borasio, P. G., Capuzzo, A., Ferreti, M. E., Trevisani, A. & Perri, V. (1982). Electrophysiological evidence for a PGE-mediated presynaptic control of acetylcholine output in the guinea-pig superior cervical ganglion. *Brain Research* **236**, 383-391.

Bennett, G. J. & Mayer, D. J. (1979). Inhibition of spinal cord interneurons by narcotic microinjection and focal electrical stimulation in the periaqueductal central gray matter. *Brain Research* **172**, 243-257.

Bessou, P. & Perl, E. R. (1969). Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *Journal of Neurophysiology* **32**, 1025-1043.

Birrell, G. J. & McQueen, D. S. (1993). The effects of capsaicin, bradykinin, PGE2 and cicaprost on the discharge of articular sensory receptors in vitro. *Brain Research* **611**, 103-

107.

Birrell, G. J., McQueen, D. S., Iggo, A. & Grubb, B. D. (1993). Prostanoid-induced potentiation of the excitatory and sensitizing effects of bradykinin on articular mechanonociceptors in the rat ankle joint. *Neuroscience* **54**, 537-544.

Blumberg, H. & Jänig, W. (1984). Discharge pattern of afferent fibers from a neuroma. *Pain* **20**, 335-353.

Brandt, M., Gullis, R. J., Fischer, K., Buchen, C., Hamprecht, B., Moräder, L. & Wünsch, E. (1976). Enkephalin regulates the levels of cyclic nucleotides in neuroblastoma x glioma hybrid cells. Nature **262**, 311-312.

Cameron, A. A., Khan, I. A., Westlund, K. N. & Willis, W. D. (1995). The efferent projections of the periaqueductal gray in the rat: a *phaseolus vulgaris*-leucoagglutinin study. II. descending projections. *Journal of Comparative Neurology* **351**, 585-601.

Campbell, J. N. & Meyer, R. A. (1983). Sensitization of unmyelinated nociceptive afferents in monkey varies with skin type. *Journal of Neurophysiology* **49**, 98-110.

Campbell, J. N., Meyer, R. A. & LaMotte, R. H. (1979). Sensitization of myelinated nociceptive afferents that innervate monkey hand. *Journal of Neurophysiology* **42**, 1669-1679.

Cangro, C. B., Sweetnam, P. M., Wrathall, J. R., Haser, W. B., Curthoys, N. P. & Neale, J. H. (1985). Localization of elevated glutaminase immunoreactivity in small DRG neurons. *Brain Research* **336**, 158-161.

Capogna, M., Gahwiler, B. H. & Thompson, S. M. (1993). Mechanism of μ-opioid receptor-mediated presynaptic inhibition in the rat hippocampus *in vitro*. *Journal of*

Physiology **470**, 539-558.

Carstens, E. (1982). Inhibition of spinal dorsal horn neuronal responses to noxious skin heating by medial hypothalamic stimulation in the cat. *Journal of Neurophysiology* **48**, 808-822.

Cervero, F., Iggo, A. & Molony, V. (1979). Ascending projections of nociceptor-driven Lamina I neurones in the cat. *Experimental Brain Research* **35**, 135-149.

Chahl, L. A. & Ladd, R. J. (1976). Local oedema and general excitation of cutaneous sensory receptors produced by electrical stimulation of the saphenous nerve of the rat. *Pain* **2**, 25-34.

Chang, F., Cohen, I. S., DiFrancesco, D., Rosen, M. R. & Tromba, C. (1991). Effects of protein kinase inhibitors on canine Purkinje fibre pacemaker depolarization and the pacemaker current i_f.. *Journal of Physiology* **440**, 367-384.

Chapman, L. F., Ramos, A. O., Goodell, H. & Wolff, H. G. (1961). Neurohumoral features of afferent fibers in man. *Archives of Neurology* **4**, 617-650.

Chase, M. R. & Ranson, S. W. (1914). The structure of the roots, trunk and branches of the vagus nerve. *Journal of Comparative Neurology* **24**, 31-60.

Chieng, B. & Christie, M. J. (1994a). Inhibition by opioids acting on μ-receptors of GABAergic and glutamatergic postsynaptic potentials in single rat periaqueductal gray neurons in vitro. *British Journal of Pharmacology* **113**, 303-309.

Chieng, B. & Christie, M. J. (1994b). Hyperpolarization by opioids acting on μ-receptors of a sub-population of rat periaqueductal gray neurons *in vitro*. *British Journal of Pharmacology* **113**, 121-128.

Coderre, T. J., Basbaum, A. I. & Levine, J. D. (1989). Neural control of vascular permeability: interactions between primary afferents, mast cells, and sympathetic efferents. *Journal of Neurophysiology* **62**, 48-58.

Coderre, T. J., Chan, A. K., Helms, C., Basbaum, A. I. & Levine, J. D. (1991). Increasing sympathetic nerve terminal-dependent plasma extravastion correlates with decreased arthritic joint injury in rats. *Neuroscience* **40**, 185-189.

Coderre, T. J., Gonzales, R., Goldyne, M. E., West, J. & Levine, J. D. (1990). Noxious stimulus-induced increase in spinal prostaglandin E2 is noradrenergic terminal-dependent. *Neuroscience Letters* **115**, 253-258.

Coggeshall, R. E., Hong, K. A. P., Langford, L. A., Schaible, H. -G. & Schmidt, R. F. (1983). Discharge characteristics of fine medial articular afferents at rest and during passive movements of inflamed knee joints. *Brain Research* **272**, 185-188.

Cohen, G. A., Doze, V. A. & Madison, D. V. (1992). Opioid inhibition of GABA release from presynaptic terminals of rat hippocampal interneurons. *Neuron* **9**, 325-335.

Collier, H. O. J. & Francis, D. L. (1975). Morphine abstinence is associated with increased brain cyclic AMP. *Nature* **255**, 159-161.

Collier, H. O. J. & Roy, A. C. (1974). Hypothesis: inhibition of E prostaglandin-sensitive adenyl cyclase as the mechanism of morphine analgesia. *Prostaglandins* **7**, 361-375.

Collins, W. F. Jr. , Nulsen, F. E. & Randt, C. T. (1960). Relation of peripheral nerve fiber size and sensation in man. *Archives of Neurology* **3**, 381-385.

Cruciani, R. A., Dvorkin, B., Morris, S. A., Crain, S. M. & Makman, M. H. (1993).

Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F11 neuroblastoma-sensory neuron hybrid cells. *Proceedings of the National Academy of Sciences USA* **90**, 3019-3023.

Crunkhorn, P. & Willis, A. L. (1971). Cutaneous reactions to intradermal prostaglandins. *British Journal of Pharmacology* **41**, 49-56.

Cui, M. & Nicol, G. D. (1995). Cyclic AMP mediates the prostaglandin E2-induced potentiation of bradykinin excitation in rat sensory neurons. *Neuroscience* **66**, 459-466.

Curtis, D. R., Duggan, A. W., Felix, D. & Johnston, G. A. R. (1971). Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. *Brain Research* **32**, 69-96.

Czlonkowski, A., Stein, C. & Herz, A. (1993). Peripheral mechanisms of opioid antinociception in inflammation: involvement of cytokines. *European Journal of Pharmacology* **242**, 229-235.

Denyer, J. C. & Brown, H. F. (1990). Pacemaking in rabbit isolated sino-atrial node cells during Cs^+ block of the hyperpolarization-activated current i_f.. *Journal of Physiology* **429**, 401-409.

DePaulis, A., Morgan, M. M. & Liebeskind, J. C. (1987). GABAergic modulation of the analgesic effects of morphine microinjected in the ventral PAG of the rat. *Brain Research* **136**, 223-228.

Devor, M. & Jänig, W. (1981). Activation of myelinated afferents ending in a neuroma by stimulation of the sympathetic supply in the rat. *Neuroscience Letters* **24**, 43-47.

Devor, M., Jänig, W. & Michaelis, M. (1994). Modulation of activity in dorsal root ganglion neurons by sympathetic activation in nerve-injured rats. *Journal of Neurophysiology* **71**, 38-47.

DiFrancesco, D., Ducouret, P. & Robinson, R.B. (1989). Muscarinic modulation of cardiac rate at low acetylcholine concentrations. *Science* **243**, 669-671.

DiFranceso, D. & Tortora, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature* **351**, 145-147.

Dray, A. & Bevan, S. (1993). Inflammation and hyperalgesia: highlighting the team effort. *Trends in Pharmacological Sciences* **14**, 287-290.

Dray, A., Patel, I. A., Perkins, M. N. & Rueff, A. (1992). Bradykinin-induced activation of nociceptors: receptor and mechanistic studies on the neonatal rat spinal cord-tail preparation *in vitro*. *British Journal of Pharmacology* **107**, 1129-1134.

Duce, I. R. & Keen, P. (1983). Selective uptake of [^{3}H]glutamine and [^{3}H]glutamate into neurons and satellite cells of dorsal root ganglia *in vitro*. *Neuroscience* **8**, 861-866.

Duggan, A. W., Hall, J. G. & Headley, P. M. (1976). Morphine, enkephalin and the substantia gelatinosa. *Nature* **264**, 456-458.

Duggan, A. W., Hall, J. G. & Headley, P. M. (1977a). Suppression of transmission of nociceptive impulses by morphine: selective effects of morphine administered in the region of the substantia gelatinosa. *British Journal of Pharmacology* **61**, 65-76.

Duggan, A. W., Hall, J. G. & Headley, P. M. (1977b). Enkephalins and dorsal horn neurones of the cat: effects on responses to noxious and innocuous skin stimuli. *British*

Journal of Pharmacology **61**, 399-408.

Duggan, A. W. & Johnston, G. A. R. (1970). Glutamate and related amino acids in cat spinal roots, dorsal root ganglia and peripheral nerves. *Journal of Neurochemistry* **17**, 1205-1208.

Duggan, A. W. & North, R. A. (1984). Electrophysiology of opioids. *Pharmacological Reviews* **35**, 219-281.

Dun, N. J. (1980). Inhibition of ACh release by prostaglandin E1 in the rabbit superior cervical ganglion. *Neuropharmacology* **19**, 1137-1140.

Eccles, J. C., Eccles, R. M. & Magni, F. (1961). Central inhibitory action attributable to presynaptic depolarization produced by muscle afferent volleys. *Journal of Physiology* **159**, 147-166.

Eickhoff, R., Handwerker, J.O., McQueen, D.S. & Schick, E. (1978). Noxious and tactile input to medial structures of midbrain and pons in the rat. *Pain* **5**, 99-113.

Engel, D. (1941). The influence of the sympathetic nervous system on capillary permeability. *Journal of Physiology* **99**, 161-181.

Fan, S. -F., Shen, K., -F. & Crain, S. M. (1991). Opioids at low concentration decrease openings of K^+ channels in sensory ganglion neurons. *Brain Research* **558**, 166-170.

Fan, S. -F., Shen, K., -F. & Crain, S. M. (1993). μ and δ opioid agonists at low concentrations decrease voltage-dependent K^+ currents in F11 neuroblastoma x DRG neuron hybrid cells via cholera toxin-sensitive receptors. *Brain Research* **605**, 214-220.

Fang, F. G., Haws, C. M., Drasner, K., Williamson, A. & Fields, H. L. (1989). Opioid peptides (DAGO-enkephalin, dynorphin A (1-13), BAM 22P) microinjected into the rat brainstem: comparison of their antinociceptive effect and their effect on neuronal firing in the rostral ventromedial medulla. *Brain Research* **501**, 116-128.

Fardin, V., Oliveras, J. -L. & Besson, J. -M. (1984). A reinvestigation of the analgesic effects induced by stimulation of the periaqueductal gray matter in the rat. II. Differential characteristics of the analgesia induced by ventral and dorsal PAG stimulation. *Brain Research* **306**, 125-139.

Ferreira, S. H., Molina, N. & Vettore, O. (1982). Prostaglandin hyperalgesia, V: a peripheral analgesic receptor for opiates. *Prostaglandins* **23**, 53-60.

Ferreira, S. H. & Nakamura, M. (1979). I - Prostaglandin hyperalgesia, a cAMP/Ca^{+2} dependent process. *Prostaglandins* **18**, 179-190.

Ferreira, S. H. & Nakamura, M. (1979). II - Prostaglandin hyperalgesia: the peripheral analgesic activity of morphine, enkephalins and opioid antagonists. *Prostaglandins* **18**, 191-200.

Fields, H. L., Barbaro, N. M. & Heinricher, M. M. (1988). Brain stem neuronal circuitry underlying the antinociceptive action of opiates. *Progress in Brain Research* **77**, 245-257.

Fields, H. L., Clanton, C. H. & Anderson, S. D. (1977). Somatosensory properties of spinoreticular neurons in the cat. *Brain Research* **120**, 49-66.

Fields, H. L., Emson, P. C., Leigh, B. K., Gilbert, R. F. T. & Iversen, L. L. (1980). Multiple opiate receptor sites on primary afferent fibres. *Nature* **284**, 351-353.

Fields, H. L., Vanegas, H., Hentall, I. D. & Zorman, G. (1983). Evidence that disinhibition of brain stem neurons contributes to morphine analgesia. *Nature* **306**, 684-686.

Fitzgerald, M. (1979). The spread of sensitization of polymodal nociceptors in the rabbit from nearby injury and by antidromic nerve stimulation. *Journal of Physiology* **297**, 207-216.

Fleetwood-Walker, S. M., Hope, P. J., Mitchell, R., El-Yassir, N. & Molony, V. (1988). The influence of opioid receptor subtypes on the processing of nociceptive inputs in the spinal dorsal horn of the cat. *Brain Research* **451**, 213-226.

Gamse, R., Holzer, P. & Lembeck, F. (1979). Indirect evidence for presynaptic location of opiate receptors on chemosensitive primary sensory neurones. *Naunyn-Schmiedeberg's Archives of Pharmacology* **308**, 281-285.

Gamse, R. & Saria, A. (1985). Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. *European Journal of Pharmacology* **114**, 61-66.

Gallego, R. & Eyzaguirre, C. (1978). Membrane and action potential characteristics of A and C nodose ganglion cells studied in whole ganglia and in tissue slices. *Journal of Neurophysiology* **41**, 1217-1232.

Giesler, G. J. Jr., Cannon, J. T., Urca, G. & Liebeskind, J. C. (1978). Long ascending projections from substantia gelatinosa rolandi and the subjacent dorsal horn in the rat. *Science* **202**, 984-986.

Glazer, E. J. & Basbaum, A. I. (1981). Immunohistochemical localization of leucine-enkephalin in the spinal cord of the cat: enkephalin-containing marginal neurons and pain

modulation. *Journal of Comparative Neurology* **196**, 377-389.

Goldyne, M. E., Winkelmann, R. K. & Ryan, R. J. (1973). Prostaglandin activity in human cutaneous inflammation: detection by radioimmunoassay. *Prostaglandins* **4**, 737-749.

Gonzales, R., Goldyne, M. E., Taiwo, Y. O. & Levine, J. D. (1989). Production of hyperalgesic prostaglandins by sympathetic postganglionic neurons. *Journal of Neurochemistry* **53**, 1595-1598.

Green, K.L. (1974). Role of endogenous catecholmines in the anti-inflammatory activity of α-adrenoceptor blocking agents. *British Journal of Pharmacology* **51**, 45-53.

Green, P. G. & Levine, J. D. (1992). δ- and κ-opioid agonists inhibit plasma extravasation induced by bradykinin in the knee joint of the rat. *Neuroscience* **49**, 129-133.

Green, P. G., Luo, J., Heller, P. & Levine, J. D. (1993a). Modulation of bradykinin-induced plasma extravasation in the rat knee joint by sympathetic co-transmitters. *Neuroscience* **52**, 451-458.

Green, P. G., Luo, J., Heller, P. H. & Levine, J. D. (1993b). Further substantiation of a significant role for the sympathetic nervous system in inflammation. *Neuroscience* **55**, 1037-1043.

Green, P. G., Luo, J., Heller, P. H. & Levine, J. D. (1994). Effect of E-type prostaglandins on bradykinin-induced plasma extravasation in the knee joint of the rat. *European Journal of Pharmacology* **252**, 127-132.

Gross, R. A. & Macdonald, R. L. (1987). Dynorphin A selectively reduces a large

transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture. *Proceedings of the National Academy of Sciences USA* **84**, 5469-5473.

Gross, R. A., Moises, H. C., Uhler, M. D. & Macdonald, R. L. (1990). Dynorphin A and cAMP-dependent protein kinase independently regulate neuronal calcium currents. *Proceedings of the National Academy of Science USA* **87**, 7025-7029.

Grudt, T. J. & Williams, J. T. (1993). κ-Opioid receptors also increase potassium conductance. *Proceedings of the National Academy of Science USA* **90**, 11420-11432.

Grudt, T. J. & Williams, J. T. (1994). μ-opioid agonists inhibit spinal trigeminal substantia gelatinosa neurons in guinea pig and rat. *Journal of Neuroscience* **14**, 1646-1654.

Grudt, T. J. & Williams, J. T. (1995). Opioid receptors and the regulation of ion conductances. *Reviews in the Neurosciences*, in press.

Guilbaud, G., Le Bars, D. & Besson, J. M. (1976). Bradykinin as a tool in neurophysiological studies of pain mechanisms. *Advances in Pain Research and Therapy* **1**, 67-73.

Handwerker, H. O. (1976). Influences of algogenic substances and prostaglandins on the discharges of unmyelinated cutaneous nerve fibers identified as nociceptors. *Advances in Pain Research and Therapy* **1**, 41-45.

Handwerker, H. O., Iggo, A. & Zimmerman, M. (1975). Segmental and supraspinal actions on dorsal horn neurons responding to noxious and non-noxious skin stimuli. *Pain* **1**, 147-165.

Harper, A. A. & Lawson, S. N. (1985a). Conduction velocity is related to morphological

cell type in rat dorsal root ganglion neurones. *Journal of Physiology* **359**, 31-46.

Harper, A. A. & Lawson, S. N. (1985b). Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. *Journal of Physiology* **359**, 47-63.

Hassan, A. H. S., Ableitner, A., Stein, C. & Herz, A. (1993). Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. *Neuroscience* **55**, 185-195.

Head, H., Rivers, W. H. R. & Sherren, J. (1905). The afferent nervous system from a new aspect. *Brain* **28**, 99-115.

Head, H. & Thompson, T. (1906). The grouping of afferent impulses within the spinal cord. *Brain* **29**, 537-645.

Heinricher, M. M., Cheng, Z. -F. & Fields, H. L. (1987). Evidence for two classes of nociceptive modulating neurons in the periaqueductal gray. *Journal of Neuroscience* **7**, 271-278.

Heinricher, M. M., Morgan, M. M., Tortorici, V. & Fields, H. L. (1994). Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventral medulla. *Neuroscience* **63**, 279-288.

Heinricher, M. M. & Tortorici, V. (1994). Interference with GABA transmission in the rostral ventral medulla: Disinhibition of off-cells as a central mechanism in nociceptive modulation. *Neuroscience* **63**, 533-546.

Hentall, I. D. & Fields, H. L. (1983). Actions of opiates, substance P, and serotonin on the excitability of primary afferent terminals and observations on interneuronal activity in

the neonatal rat's dorsal horn in vitro. *Neuroscience* **9**, 521-528.

Heppelmann, B., Pfeffer, A., Schaible, H. -G. & Schmidt, R. F. (1986). Effects of acetylsalicyclic acid and indomethacin on single groups III and IV sensory units from acutely inflamed joints. *Pain* **26**, 337-351.

Heppelmann, B., Schaible, H.-G. & Schmidt, R. F. (1985). Effects of prostaglandins E1 and E2 on the mechanosensitivity of group III afferents from normal and inflamed cat knee joints. *Advances in Pain Research and Therapy* **9**, 91-101.

Higashi, H., Shinnick-Gallagher, P. & Gallagher, J. P. (1982). Morphine enhances and depresses Ca^{+2}-dependent responses in visceral primary afferent neurons. *Brain Research* **251**, 186-191.

Hingtgen, C. M., Waite, K. J. & Vasko, M. R. (1995). Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3',5'-cyclic monophosphate transduction cascade. *Journal of Neuroscience* **15**, 5411-5419.

Hirota, N., Kuraishi, Y., Hino, Y., Sato, Y., Satoh, M. & Takagi, H. (1985). Met-enkephalin and morphine but not dynorphin inhibit noxious stimuli-induced release of substance P from rabbit dorsal horn *in situ*. *Neuropharmacology* **24**, 567-570.

Hodge, C.J. Apkarian, A.V., Stevens, R. Vogelsang, G. & Wisnicki, H.J. (1981). Locus coeruleus modulation of dorsal horn unit reponses to cutaneous stimulation. *Brain Research* **204**, 415-420.

Holzer, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* **24**, 739-768.

Hori, Y., Endo, K. & Takahashi, T. (1992). Presynaptic inhibitory action of enkephalin on excitatory transmission in superficial dorsal horn of rat spinal cord. *Journal of Physiology* **450**, 673-685.

Hosobuchi, Y., Adams, J. E. & Linchitz, R. (1977). Pain relief by electrical stimulation of the central gray matter in humans and its reversal by naloxone. *Science* **197**, 183-186.

Howland, B., Lettvin, J. Y., McCulloch, W. S., Pitts, W. & Wall, P. D. (1955). Reflex inhibition by dorsal root interaction. *Journal of Physiology* **18**, 1-17.

Iggo, A. (1963). New specific sensory structures in hairy skin. *Acta Neurovegetativa* **24**, 175-180.

Janscó, N., Janscó-Gábor, A. & Szolcsányi, J. (1967). Direct evidence for neurogenic inflammation and its prevention by denervation and by treatment with capsaicin. *British Journal of Pharmacology and Chemotherapy* **31**, 138-151.

Jensen, T. S. & Yaksh, T. L. (1986). III. Comparison of the antinociceptive action of mu and delta opioid receptor ligands in the periaqueductal gray matter, medial and paramedial ventral medulla in the rat as studied by the microinjection technique. *Brain Research* **372**, 301-312.

Jensen, T. S. & Yaksh, T. L. (1989). Comparison of the antinociceptive effect of morphine and glutamate at coincidental sites in the periaqueductal gray and medial medulla in rats. *Brain Research* **476**, 1-9.

Johansson, O., Hökfelt, T., Pernow, B., Jeffcoate, S. L., White, N., Steinbusch, H. W. M., Verhofstad, A. A. J., Emson, P. C. & Spindel, E. (1981). Immunohistochemical support for three putative transmitters in one neuron: coexistence of 5-hydroxytryptamine,

substance P- and thyrotropin releasing hormone-like immunoreactivity in medullary neurons projecting to the spinal cord. *Neuroscience* **6**, 1857-1881.

Johnson, S. M. & Duggan, A. W. (1981). Evidence that the opiate receptors of the substantia gelatinosa contribute to the depression, by intravenous morphine, of the spinal transmission of impulses in unmyelinated primary afferents. *Brain Research* **207**, 223-228.

Johnson, S. W. & North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *Journal of Neuroscience* **12**, 483-488.

Jones, S.L. & Gebhart, G.F. (1986). Characterization of coeruleospinal inhibition of the nociceptivetail-flick reflex in the rat: mediation by spinal α_2-adrenoceptors. *Brain Research* **364**, 315-330.

Kalia, M. & Mesulam, M. -M. (1980). Brain stem projections of sensory and motor components of the vagus nerve in the cat: 1. The cervical vagus and nodose ganglion. *Journal of Comparative Neurology* **193**, 435-465.

Kangrga, I. & Randi´c, M. (1991). Outflow of endogenous aspartate and glutamate from the rat spinal dorsal horn in vitro by activation of low- and high-threshold primary afferent fibers. Modulation by opioids. *Brain Research* **553**, 347-352.

Kenins, P. (1980). Identification of the unmyelinated sensory nerves which evoke plasma extravasation in response to antidromic stimulation. *Neuroscience Letters* **16**, 161-166.

Kessler, W., Kirchhoff, C., Reeh, P. W. & Handwerker, H. O. (1992). Excitation of cutaneous afferent nerve endings in vitro by a combination of inflammatory mediators and conditioning effect of substance P. *Experimental Brain Research* **91**, 467-476.

Khasar, S. G., Green, P. G. & Levine, J. D. (1993). Comparison of intradermal and subcutaneous hyperalgesic effects of inflammatory mediators in the rat. *Neuroscience Letters* **153**, 215-218.

Khasar, S. G., Ho, T., Green, P. G. & Levine, J. D. (1994). Comparison of prostaglandin E1- and prostaglandin E2-induced hyperalgesia in the rat. *Neuroscience* **62**, 345-350.

Lacey, M. G., Mercuri, N. B. & North, R. A. (1989). Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *Journal of Neuroscience* **9**, 1233-1241.

Laduron, P. M. (1984). Axonal transport of opiate receptors in capsaicin-sensitive neurones. *Brain Research* **294**, 157-160.

LaMotte, C., Pert, C. B. & Snyder, S. H. (1976). Opiate receptor binding in primate spinal cord: distribution and changes after dorsal root section. Brain Research **112**, 407-412.

LaMotte, R. H., Thalhammer, J. G. & Robinson, C. J. (1983). Peripheral neural correlates of magnitude of cutaneous pain and hyperalgesia: a comparison of neural events in monkey with sensory judgements in human. *Journal of Neurophysiology* **50**, 1-26.

LaMotte, R. H., Thalhammer, J. G., Torebjörk, H. E. & Robinson, C. J. (1982). Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat. *Pain* **2**, 765-781.

Langley, J. N. (1923). Antidromic action. *Journal of Physiology* 57, 428-446.

Law, P. Y., Hom, D. S. & Loh, H. H. (1982). Loss of opiate receptor activity in

neuroblastoma x glioma NG108-15 hybrid cells after chronic opiate treatment. *Molecular Pharmacology* **22**, 1-4.

Le Bars, D., Dickenson, A. H. & Besson, J. M. (1980). Microinjection of morphine within nucleus raphe magnus and dorsal horn neurone activities related to nociception in the rat. *Brain Research* **189**, 467-481.

Le Bars, D., Menétrey, D., Conseiller, C. & Besson, J. M. (1975). Depressive effects of morphine on lamina V cell activities in the dorsal horn of the spinal cat. *Brain Research* **98**, 261-277.

Lembeck, F. (1983). Sir Thomas Lewis's nocifensor system, histamine and substance-P-containing primary afferent nerves. *Trends in Neurological Sciences* **6**, 106-108.

Lembeck, F. & Donnerer, J. (1985). Opioid control of the function of primary afferent substance P fibres. *European Journal of Pharmacology* **114**, 241-246.

Lembeck, F., Donnerer, J. & Barthó, L. (1982). Inhibition of neurogenic vasodilation and plasma extravasation by substance P antagonists, somatostatin and (D-Met^2, Pro^5) enkephalinamide. *European Journal of Pharmacology* **85**, 171-176.

Lembeck, F., Popper, H. & Juan, H. (1976). Release of prostaglandins by bradykinin as an intrinsic mechanism of its algesic effect. *Nauyn-Schmeideberg's Archives of Pharmacology* **294**, 69-73.

Levine, J. D. & Taiwo, Y. O. (1989). Involvement of the mu-opiate receptor in peripheral analgesia. *Neuroscience* **32**, 571-575.

Levine, J. D., Taiwo, Y. O., Collins, S. D. & Tam, J. K. (1986). Noradrenaline

hyperalgesia is mediated through interaction with sympathetic postganglionic neurone terminals rather than activation of primary afferent nociceptors. *Nature* **323**, 158-160.

Levy, R. A. (1974). GABA: a direct depolarizing action at the mammalian primary afferent terminal. *Brain Research* **76**, 155-160.

Lewis, J. W., Baldrighi, G. & Akil, H. (1987). A possible interface between autonomic function and pain control: opioid analgesia and the nucleus tractus solitarius. *Brain Research* **424**, 65-70.

Lewis, T. (1936). Experiments relating to cutaneous hyperalgesia and its spread through somatic nerves. *Clinical Science* **2**, 373-423.

Lewis, V. A. & Gebhart, G. F. (1977). Evaluation of the periaqueductal central gray as a morphine-specific locus of action and examination of morphine-induced and stimulation-produced analgesia at coincident PAG loci. *Brain Research* **124**, 283-303.

Light, A. R., Casale, E. J. & Menétrey, D. M. (1986). The effects of focal stimulation in nucleus raphe magnus and periaqueductal gray on intracellularly recorded neurons in spinal laminae I and II. *Journal of Neurophysiology* **56**, 555-571.

Lin, Q., Peng, Y. & Willis, W. D. (1994). Glycine and $GABA_A$ antagonists reduce the inhibition of primate spinothalamic tract neurons produced by stimulation in the periaqueductal gray. *Brain Research* **654**, 286-302.

Loewenstein, W. R. (1963). Excitation processes in a receptor membrane. *Acta Neurovegetativa* **24**, 184-207.

Lynn, B. (1977). Cutaneous hyperalgesia. *British Medical Bulletin* **33**, 103-108.

Macdonald, R. L. & Nelson, P. G. (1978). Specific-opiate-induced depression of transmitter release from dorsal root ganglion cells in culture. *Science* **199**, 1449-1450.

MacLean, D. B., Bennett, B., Morris, M. & Wheeler, F. B. (1989). Differential regulation of calcitonin gene-related peptide and substance P in cultured neonatal rat vagal sensory neurons. *Brain Research* **478**, 349-355.

Madison, D. V. & Nicoll, R. A. (1988). Enkephalin hyperpolarizes interneurons in the rat hippocampus. *Journal of Physiology* **398**, 123-130.

Maekawa, K., Minami, M., Yabuuchi, K., Toya, T., Katao, Y., Hosoi, Y., Onogi, T. & Satoh, M. (1994). In situ hybridization study of μ- and κ-opioid receptor mRNAs in the rat spinal cord and dorsal root ganglia. *Neuroscience Letters* **168**, 97-100.

Makman, M. H., Dvorkin, B. & Crain, S. M. (1988). Modulation of adenylyl cyclase activity of mouse spinal cord-ganglion explants by opioids, serotonin and pertussis toxin. *Brain Research* **445**, 303-313.

Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H. & Watson, S. J. (1987). Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *Journal of Neuroscience* 7, 2445-2464.

Martin, H. A., Basbaum, A. I., Kwiat, G. C., Goetzl, E. J. & Levine, J. D. (1987). Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-δ mechanonociceptors in the hairy skin of rat hindlimbs. *Neuroscience* **22**, 651-659.

Mayer, D. J. & Liebeskind, J. C. (1974). Pain reduction by focal electrical stimulation of the brain: an anatomical and behavioral analysis. *Brain Research* **68**, 73-93.

McCormick, D.A. & Pape, H-C. (1990). Noradrenergic and serotonergic modulation of a

hyperpolarization-activated cation current in thalamic relay neurons. *Journal of Physiology* **431**, 319-342.

McLachlan, E. M., Jänig, W., Devor, M. & Michaelis, M. (1993). Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. *Nature* **363**, 543-546.

McLaughlin, B. J., Barber, R., Saito, K., Roberts, E. & Wu, J. Y. (1975). Immunocytochemical localization of glutamate decarboxylase in rat spinal cord. *Journal of Comparative Neurology* **164**, 305-322.

McLean, M. J., Bennett, P. B. & Thomas, R. M. (1988). Subtypes of dorsal root ganglion neurons based on different inward currents as measured by whole-cell voltage-clamp. *Molecular and Cellular Biochemistry* **80**, 95-107.

Melzack, R. & Wall, P. D. (1965). Pain mechanisms: a new theory. *Science* **150**, 971-983.

Mendell, L. M. (1966). Physiological properties of unmyelinated fiber projection to the spinal cord. *Experimental Neurology* **16**, 316-332.

Mendell, L. M. & Wall, P. D. (1964). Presynaptic hyperpolarization: a role for fine afferent fibres. *Journal of Physiology* **172**, 274-294.

Mendell, L. M. & Wall, P. D. (1965). Responses of single dorsal cord cells to peripheral cutaneous unmyelinated fibers. *Nature* **206**, 97-99.

Mense, S. (1981). Sensitization of group IV muscle receptors to bradykinin by 5-hydroxytryptamine and prostaglandin E2. *Brain Research* **225**, 95-105.

Meyer, R. A. & Campbell, J. N. (1981). Myelinated nociceptive afferents account for the

hyperalgesia that follows a burn to the hand. *Science* **213**, 1527-1529.

Millhorn, D. E., Hökfelt, T., Seroogy, K., Oertel, W., Verhofstad, A. A. J. & Wu, J. -Y. (1987). Immunohistochemical evidence for colocalization of x-aminobutyric acid and serotonin in neurons of the ventral medulla oblongata projecting to the spinal cord. *Brain Research* **410**, 179-185.

Millhorn, D. E., Hökfelt, T., Terenius, L., Buchan, A. & Brown, J. C. (1987). Somatostatin- and enkephalin-like immunoreactivities are frequently colocalized in neurons in the caudal brain stem of rat. *Experimental Brain Research* **67**, 420-428.

Moncada, S., Ferreira, S. H. & Vane, J. R. (1973). Prostaglandins, aspirin-like drugs and the oedema of inflammation. *Nature* **246**, 217-218.

Moreau, J. -L. & Fields, H. L. (1986). Evidence for GABA involvement in midbrain control of medullary neurons that modulate nociceptive transmission. *Brain Research* **397**, 37-46.

Morgan, M. M., Heinricher, M. M. & Fields, H. L. (1992). Circuitry linking opioid-sensitive nociceptive modulatory systems in the PAG and spinal cord with rostral ventromedial medulla. *Neuroscience* **47**, 863-871.

Mudge, A. W., Leeman, S. E. & Fischbach, G. D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proceedings of the National Academy of Sciences USA* **76**, 526-530.

Nafe, J. P. (1929). A quantitative theory of feeling. *Journal of General Psychology* **2**, 199-210.

Nathan, P. W. (1976). The gate control theory of pain: a critical review. *Brain* **99**, 123-

158.

Neto, F. R. (1978). The depolarizing action of 5-HT on mammalian non-myelinated nerve fibres. *European Journal of Pharmacology* **49**, 351-356.

Nicol, G. D. & Cui, M. (1994). Enhancement by prostaglandin E2 of bradykinin activation of embryonic rat sensory neurones. *Journal of Physiology* **480**, 485-492.

Nicol, G. D., Klingberg, D. K. & Vasko, M. R. (1992). Prostaglandin E2 increases calcium conductance and stimulates release of Substance P in avian sensory neurons. *Journal of Neuroscience* **12**, 1917-1927.

Nicoll, R. A., Alger, B. E. & Jahr, C. E. (1980). Enkephalin blocks inhibitory pathways in the vertebrate CNS. *Nature* **287**, 22-25.

Ninkovic, M., Hunt, S. P. & Gleave, J. R. W. (1982). Localization of opiate and histomine H1-receptors in the primate sensory ganglia and spinal cord. *Brain Research* **241**, 197-206.

North, R. A. & Williams, J. T. (1983). How do opiates inhibit neurotransmitter release. *Trends in Neurological Sciences* **4**, 337-339.

North, R. A., Williams, J. T., Surprenant, A. & Christie, M. J. (1987). μ- and δ-receptors belong to a family of receptors that are coupled to potassium channels. *Proceedings of the National Academy of Sciences USA* **84**, 5487-5491.

Olianas, M. C. & Onali, P. (1993). Synergistic interaction of muscarinic and opioid receptors with Gs-linked neurotransmitter receptors to stimulate adenylyl cyclase activity of rat olfactory bulb. *Journal of Neurochemistry* **61**, 2183-2190.

Oliveras, J. L., Redjemi, F., Guilbaud, G. & Besson, J. M. (1975). Analgesia induced by

electrical stimulation of the inferior centralis nucleus of the raphe in the cat. *Pain* **1**, 139-145.

Onali, P. & Olianas, M. C. (1991). Naturally occurring opioid receptor agonists stimulate adenylate cyclase activity in rat olfactory bulb. *Molecular Pharmacology* **39**, 436-441.

Otsuka, M. & Yoshioka, K. (1993). Neurotransmitter functions of mammalian tachykinins. *Physiological Reviews* **73**, 229-308.

Ouseph, A. K., Khasar, S. G. & Levine, J. D. (1995). Multiple second messenger systems act sequentially to mediate rolipram-induced prolongation of prostaglandin E2-induced mechanical hyperalgesia in the rat. *Neuroscience* **64**, 769-776.

Owman, C. & Santini, M. (1966). Adrenergic nerves in spinal ganglia of the cat. *Acta Physiologica Scandinavia* **68**, 127-128.

Pan, Z. Z., Williams, J. T. & Osborne, P. B. (1990). Opioid actions on single nucleus raphe magnus neurons from rat and guinea pig in vitro. *Journal of Physiology* **427**, 519-532.

Parsons, C. G. & Herz, A. (1990). Peripheral opioid receptors mediating antinociception in inflammation. Evidence for activation by enkephalin-like opioid peptides after cold water swim stress. *Journal of Pharmacology and Experimental Therapeutics* **255**, 795-802.

Parsons, C. G., Czlonkowski, A., Stein, C. & Herz, A. (1990). Peripheral opioid receptors mediating antinociception in inflammation. Activation by endogenous opioids and role of the pituitary-adrenal axis.*Pain* **41**, 81-93.

Perl, E. R. (1968). Myelinated afferent fibres innervating the primate skin and their response to noxious stimuli. *Journal of Physiology* **197**, 593-615.

Perl, E. R. (1976). Sensitization of nociceptors and its relation to sensation. *Advances in Pain Research and Therapy* **1**, 17-28.

Piercey, M. F., Dobry, P. J. K., Schroeder, L. A. & Einspahr, F. J. (1981). Behavioral evidence that substance P may be a spinal cord sensory neurotransmitter. *Brain Research* **210**, 407-412.

Pitchford, S. & Levine, J. D. (1991). Prostaglandins sensitize nociceptors in cell culture. *Neuroscience Letters* **132**, 105-108.

Price, D. D., Hull, C. D. & Buchwald, N. A. (1971). Intracellular responses of dorsal horn cells to cutaneous and sural nerve A and C fiber stimuli. *Experimental Neurology* **33**, 291-309.

Price, D. D. & Wagman, I. H. (1970). Physiological roles of A and C fiber inputs to the spinal dorsal horn of *Macaca mulatta*. *Experimental Neurology* **29**, 383-399.

Przewlocka, B., Dziedzicka, M., Lason, W. & Przewlocki, R. (1991). Differential effects of opioid receptor agonists on nociception and cAMP level in the spinal cord of monoarthritic rats. *Life Sciences* **50**, 45-54.

Quigg, M., Elfvin, L. -G. & Aldskogius, H. (1990). Anterograde transsynaptic transport of WGA-HRP from spinal afferents to postganglionic sympathetic cells of the stellate ganglion of the guinea pig. *Brain Research* **518**, 173-178.

Randi´c, M. & Mileti´c, V. (1977). Effect of substance P in cat dorsal horn neurones activated by noxious stimuli. *Brain Research* **128**, 164-169.

Ranson, S. W. (1912). The structure of the spinal ganglia and of the spinal nerves.

Journal of Comparative Neurology **22**, 159-175.

Ranson, S. W. (1913). The course within the spinal cord of the non-medullated fibers of the dorsal roots: a study of Lissauer's tract in the cat. *Journal of Comparative Neurology* **23**, 259-274.

Ranson, S. W. (1914). An experimental study of Lissauer's tract and the dorsal roots. *Journal of Comparative Neurology* **24**, 531-545.

Ranson, S. W. (1915). Unmyelinated nerve-fibers as conductors of protopathic sensation. *Brain* **38**, 381-389.

Ranson, S. W. & Billingsley, P. R. (1916). The conduction of painful afferent impulses in the spinal nerves. *American Journal of Physiology* **40**, 571-584.

Reeh, P. W., Kocher, L. & Jung, S. (1986). Does neurogenic inflammation alter the sensitivity of unmyelinated nociceptors in the rat? *Brain Research* **384**, 42-50.

Reichling, D. B. & Basbaum, A. I. (1990a). Contribution of brainstem GABAergic circuitry to descending antinociceptive controls. I. GABA-immunoreactive projection neurons in the periaqueductal gray and the nucleus raphe magnus. *Journal of Comparative Neurology* **302**, 370-377.

Reichling, D. B. & Basbaum, A. I. (1990b). Contribution of brainstem GABAergic circuitry to descending antinociceptive controls. II. Electron microscopy immunocytochemical evidence of GABAergic control over the projection from the periaqueductal gray to the nucleus raphe magnus in the rat. *Journal of Comparative Neurology* **302**, 378-393.

Reichling, D. B., Kwait, G. C. & Basbaum, A. I. (1988). Anatomy, physiology and

pharmacology of the periaqueductal gray contribution to antinociceptive controls. *Progress in Brain Research* **77**, 31-46.

Repkin, A. H., Wolf, P. & Anderson, E. G. (1976). Non-GABA mediated primary afferent depolarization. *Brain Research* **117**, 147-152.

Rexed, B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. *Journal of Comparative Neurology* **96**, 415-466.

Roberts, W. J. & Levitt, G. R. (1982). Histochemical evidence for sympathetic innervation of hair receptor afferents in cat skin. *Journal of Comparative Neurology* **210**, 204-209.

Ruda, M. A. (1982). Opiates and pain pathways: demonstration of enkephalin synapses on dorsal horn projection neurons. *Science* **215**, 1523-1525.

Rueff, A. & Dray, A. (1993). Sensitization of peripheral afferent fibres in the *in vitro* neonatal rat spinal cord-tail by bradykinin and prostaglandins. *Neuroscience* **54**, 527-535.

Russell, N. J. W., Schaible, H. -G. & Schmidt, R. F. (1987). Opiates inhibit the discharges of fine afferent units from the inflamed knee joint of the cat. *Neuroscience Letters* **76**, 107-112.

Saria, A., Martling, C. -R., Yan, Z., Theodorsson-Norheim, E., Gamse, R. & Lundberg, J. M. (1988). Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *American Review of Respiratory Disorders* **137**, 1330-1335.

Sato, J. & Perl, E. R. (1991). Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury. Science **251**, 1608-1610.

Sato, J., Suzuki, S., Iseki, T. & Kumazawa, T. (1993). Adrenergic excitation of cutaneous nociceptors in chronically inflamed rats. *Neuroscience Letters* **164**, 225-228.

Schaible, H. -G. & Schmidt, R. F. (1985). Effects of an experimental arthritis on the sensory properties of fine articular afferent units. *Journal of Neurophysiology* **54**, 1109-1122.

Schaible, H. -G. & Schmidt, R. F. (1988). Excitation and sensitization of fine articular afferents from cat's knee joint by prostaglandin E2. *Journal of Physiology* **403**, 91-104.

Schepelmann, K., Meßlinger, K., Schaible, H. -G. & Schmidt, R. F. (1992). Inflammatory mediators and nociception in the joint: excitation and sensitization of slowly conducting afferent fibers of cat's knee by prostaglandin I2. *Neuroscience* **50**, 237-247.

Schepelmann, K., Meßlinger, K., Schaible, H. -G. & Schmidt, R. F. (1995). The opioid antagonist naloxone does not alter discharges of nociceptive afferents from the acutely inflamed knee joint of the cat. *Neuroscience Letters* **187**, 212-214.

Schneider, S. P. & Perl, E. R. (1985). Selective excitation of neurons in the mammalian spinal dorsal horn by aspartate and glutamate in vitro: correlation with location and excitatory input. *Brain Research* **360**, 339-343.

Schroeder, J. E., Fischbach, P. S., Zheng, D. & McCleskey, E. W. (1991). Activation of mu opioid receptors inhibits transient high and low threshold Ca^{2+} currents but spares a sustained current. *Neuron* **6**, 13-20.

Senami, M., Aoki, M., Kitahata, L. K.,Collins, J. G., Kumeta, Y. & Murata, K. (1986). Lack of opiate effects on cat C polymodal nociceptive fibers. *Pain* **27**, 81-90.

Sharma, S. K., Nirenberg, M. & Klee, W. A. (1975). Morphine receptors as regulators of adenylate cyclase activity. *Proceedings of the National Academy of Sciences USA* **72**, 590-594.

Shea, V. K. & Perl, E. R. (1985). Failure of sympathetic stimulation to affect responsiveness of rabbit polymodal nociceptors. *Journal of Neurophysiology* **54**, 513-519.

Shefner, S. A., North, R. A. & Zukin, R. S. (1981). Opiate effects on rabbit vagus nerve: electrophysiology and radioligand binding. *Brain Research* **221**, 109-116.

Shen, K. -F. & Crain, S. M. (1989). Dual opioid modulation of the action potential duration of mouse dorsal root ganglion neurons in culture. *Brain Research* **491**, 227-242.

Shir, Y. & Seltzer, Z. (1991). Effects of sympathectomy in a model of causalgiform pain produced by partial sciatic nerve injury in rats. *Pain* **45**, 309-320.

Sicuteri, F., Fanciullacci, M., Franchi, G. & Del Bianco, P. L. (1965). Serotonin - bradykinin potentiation on the pain receptors in man. *Life Sciences* **4**, 309-316.

Sinclair, D. C. (1955). Cutaneous sensation and the doctrine of specific energy. *Brain* **78**, 584-614.

Sinclair, D. C. & Stokes, B. A. R. (1964). The production and characteristics of "second pain". *Brain* **87**, 609-618.

Smith, D. J., Robertson, B., Monroe, P. J., Taylor, D. A., Leedham, J. A. & Cabral, J. D. Y. (1992). Opioid receptors mediating antinociception from ß-endorphin and morphine in the PAG. *Neuropharmacology* **31**, 1137-1150.

Stanfield, P. R., Nakajima, Y. & Yamaguchi, K. (1985). Substance P raises neuronal membrane excitability by reducing inward rectification. *Nature* **315**, 498-501.

Stansfield, C. E. & Wallis, D. I. (1985). Properties of visceral primary afferent neurons in the nodose ganglion of the rabbit. *Journal of Neurophysiology* **54**, 245-260.

Stein, C., Gramsch, C. & Herz, A. (1990). Intrinsic mechanisms of antinociception in inflammation: local opioid receptors and ß-endorphin. *Journal of Neuroscience* **10**, 1292-1298.

Stein, C., Hassan, A. H. S., Przewlocki, R., Gramsch, C. Peter, K. & Herz, A. (1990). Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. *Proceedings of the National Academy of Sciences* **87**, 5935-5939.

Stein, C., Millan, M. J., Shippenberg, T. S. & Herz., A. (1988). Peripheral effect of fentanyl upon nociception in inflamed tissue of the rat. *Neuroscience Letters* **84**, 225-228.

Stein, C., Millan, M. J., Shippenberg, T. S., Peter, K. & Herz, A. (1989). Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of *Mu, Delta* and *Kappa* receptors. *Journal of Pharmacology and Experimental Therapeutics* **248**, 1269-1275.

Stevens, R. T., Hodge, C. J. Jr. & Apkarian, A. V. (1983). Catecholamine varicosities in cat dorsal root ganglion and spinal ventral roots. *Brain Research* **261**, 151-154.

Szolcsányi, J. (1988). Antidromic vasodilatation and neurogenic inflammation. *Agents and Actions* **23**, 4-11.

Taiwo, Y. O., Bjerknes, L. K., Goetzl, E. J. & Levine, J. D. (1989). Mediation of primary afferent peripheral hyperalgesia by the cAMP second messenger system. *Neuroscience* **32**,

577-580.

Taiwo, Y. O., Heller, P. H. & Levine, J. D. (1992). Mediation of serotonin hyperalgesia by the cAMP second messenger system. *Neuroscience* **48**, 479-483.

Taiwo, Y. O. & Levine, J. D. (1989a). Prostaglandin effects after elimination of indirect hyperalgesic mechanisms in the skin of the rat. *Brain Research* **492**, 397-399.

Taiwo, Y. O. & Levine, J. D. (1989b). Contribution of guanine nucleotide regulatory proteins to prostaglandin hyperalgesia in the rat. *Brain Research* **492**, 400-403.

Taiwo, Y. O. & Levine, J. D. (1991a). Further confirmation of the role of adenyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neuroscience* **44**, 131-135.

Taiwo, Y. O. & Levine, J. D. (1991b). κ- and δ-opioids block sympathetically dependent hyperalgesia. *Journal of Neuroscience* **11**, 928-932.

Tang, W. -J. & Gilman, A. G. (1991). Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science* **254**, 1500-1503.

Tokimasa, T., Tsurusaki, M. & Akasu, T. (1993). Chemosensitivity of C-cells in bullfrog dorsal root ganglia to substance P and adenosine 5'-triphosphate. *Neuroscience Letters* **163**, 169-172.

Traber, J., Fischer, K., Latzin, S. & Hamprecht, B. (1975). Morphine antagonises action of prostaglandin in neuroblastoma and neuroblastoma x glioma hybrid cells. *Nature* **253**, 120-122.

Travagli, R. A. & Williams, J. T. (1995). Serotonin and noradrenaline inhibit glutamate transmission in the substantia gelatinosa of the spinal trigeminal nucleus in guinea pig. *Submitted.*

Tseng, L. L. F., Tang, R., Stackman, R., Camara, A. & Fujimoto, J. M. (1990). Brainstem sites differentially sensitive to β-endorphin and morphine for analgesia and release of met-enkephalin in anesthetized rats. *Journal of Pharmacology and Experimental Therapeutics* **253**, 930-937.

Urban, M. O. & Smith, D. J. (1994) Nuclei within the rostral ventromedial medulla mediating morphine antinociception from the periaqueductal gray. *Brain Research* **652**, 9-16.

van Praag, H. & Frenk, H. (1990). The role of glutamate in opiate descending inhibition of nociceptive spinal reflexes. *Brain Research* **524**, 101-105.

Vasko, M. R., Campbell, W. B. & Waite, K. J. (1994). Prostaglandin E2 enhances bradykinin-stimulated release of neuropeptides from rat sensory neurons in culture. *Journal of Neuroscience* **14**, 4987-4997.

Vierck, C. J. Jr. & Luck, M. M. (1979). Loss and recovery of reactivity to noxious stimuli in monkeys with primary spinothalamic cordotomies, followed by secondary and tertiary lesions of other cord sectors. *Brain* **102**, 233-248.

Waksman, G., Hamel, E., Fournié-Zaluski, M.-C. & Roques, B. P. (1986). Autoradiographic comparison of the distribution of the neutral endopeptidase "enkephalinase" and of μ and δ receptors in rat brain. *Proceedings of the National Academy of Sciences USA* **83**, 1523-1527.

Wall, P. D. (1978). The gate control theory of pain mechanisms: A re-examination and re-statement. *Brain* **101**, 1-18.

Weddell, G. (1955). Somesthesis and the chemical senses. *Annual Review of Psychology* **6**, 119-136.

Wellman, W. & Schwabe, U. (1973). Effects of prostaglandins E1, E2 and F2α on cyclic AMP levels in brain *in vivo*. *Brain Research* **59**, 371-378.

Werz, M. A. & Macdonald, R. L. (1983). Opioid peptides selective for mu- and delta-opiate receptors reduce calcium-dependent action potential duration by increasing potassium conductance. *Neuroscience Letters* **42**, 173-178.

Williams, J. T., Egan, T. M. & North, R. A. (1982). Enkephalin opens potassium channels on mammalian central neurons. *Nature* **299**, 74-77.

Williams, J. T. & Zieglgansberger, W. (1981). Mature spinal ganglion cells are not sensitive to opiate receptor mediated actions. *Neuroscience Letters* **21**, 211-216.

Williams, T. J. & Morley, J. (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature* **246**, 215-217.

Williams, T. J. & Peck, M. J. (1977). Role of prostaglandin-mediated vasodilatation in inflammation. *Nature* **270**, 530-532.

Willis, A. L. & Cornelsen, M. (1973). Repeated injection of prostaglandin E2 in rat paws induces chronic swelling and a marked decrease in pain threshold. *Prostaglandins* **3**, 353-357.

Willis, W. D., Leonard, R. B. & Kenshalo, D. R. Jr. (1978). Spinothalamic tract neurons in the substantia gelatinosa. *Science* **202**, 986-988.

Woolf, C. J. (1980). Analgesia and hyperalgesia produced in the rat by intrathecal naloxone. *Brain Research* **189**, 593-597.

Woolf, C. J. & Fitzgerald, M. (1982). Do opioid peptides mediate a presynaptic control of C-fibre transmission in the rat spinal cord? *Neuroscience Letters* **29**, 67-72.

Yaksh, T. L. (1988). Substance P release from knee joint afferent terminals: modulation by opioids. *Brain Research* **458**, 319-324.

Yaksh, T. L., Yeung, J. C. & Rudy, T. A. (1976). Systemic examination in the rat of brain sites sensitive to the direct application of morphine: observation of differential effects within the PAG. *Brain Research* **114**, 83-103.

Yaksh, T. L. & Rudy, T. A. (1976). Analgesia mediated by a direct spinal action of narcotics. *Science* **192**, 1357-1358.

Yanagisawa, M., Otsuka, M. & Garciá-Arrarás, J. E. (1986). E-type prostaglandins depolarize primary afferent neurons of the neonatal rat. *Neuroscience Letters* **68**, 351-355.

Yatani, A., Okabe, K., Codina, J., Birnbaumer, L. & Brown, A. M. (1990). Heart rate regulation by G proteins acting on the cardiac pacemaker channel. *Science* **249**, 1163-1166.

Yezierski, R. P., Bowker, R. M., Kevetter, G. A., Westlund, K. N., Coulter, J. D. & Willis, W. D. (1982). Serotonergic projections to the caudal brain stem: a double label study using horseradish preoxidase and serotonin immunocytochemstry. *Brain Research* **239**, 258-264.

Zambotti, F., Zonta, N., Parenti, M., Tommasi, R., Vicentini, L., Conci, F. & Mantegazza, P. (1982). Periaqueductal gray matter involvement in the muscimol-induced decrease of morphine antinociception. *Nauyn-Schmiedeberg's Archives of Pharmacology* **318**, 368-369.

Zarbin, M. A., Wamsley, J. K. & Kuhar, M. J. (1990). Anterograde transport of opioid receptors in rat vagus nerves and dorsal roots of spinal nerves: pharmacology and sensitivity to sodium and guanine nucleotides. *Experimental Brain Research* **81**, 267-278.

Zotterman, Y. (1963). Specificity of cutaneous afferent nerve fibers. *Acta Neurovegatativa* **24**, 181-188.

Manuscript 1:

Neuron (1994) 13:179-186

Opioid inhibition of I_h via adenylyl cyclase

By: Susan L. Ingram and John T. Williams

From: Vollum Institute and Department of
Pharmacology,
Oregon Health Sciences University,
Portland, Oregon 97201

Running title: Opioid inhibition of I_h

pages: 21

principal text: 12

figures: 6

tables: 1

Acknowledgements: This work was supported by NIH Grants from NIDA DA00141, DA08163, and DA07262. We would like to thank R. A. Travagli, N. V. Marrion and E. W. McCleskey for helpful comments on this manuscript.

Correspondence: Dr. J.T. Williams
Vollum Institute
Oregon Health Sciences University, L-474
3181 SW Sam Jackson Park Road
Portland, Oregon 97201, U.S.A.
Telephone: (503) 494-5465
Facsimile: (503) 494-6972
Internet: williamj@ohsu.edu

ABSTRACT

Opioids are coupled through G-proteins to both ion channels and adenylyl cyclase. This study describes opioid modulation of the voltage-dependent cation channel, I_h , in cultured guinea pig nodose ganglion neurons. Forskolin, PGE_2 and cAMP analogs shifted the voltage dependence of activation of I_h to more depolarized potentials and increased the inward current at -60 mV. Opioids had no effect on I_h alone, but reversed the effect of forskolin on I_h. This action of opioids was blocked by naloxone. Opioids had no effect on I_h in the presence of cAMP analogs, suggesting that modulation occurs at the level of adenylyl cyclase. The shift in voltage dependence of I_h by agents that induce inflammation (i.e. PGE_2) is one potential mechanism to mediate an increased excitability. Opioid inhibition of adenylyl cyclase and subsequent inhibition of I_h may be a mechanism by which opioids inhibit primary afferent excitability and relieve pain.

INTRODUCTION

Peripherally applied opioids cause analgesia (Ferreira and Nakamura, 1979; Ferreira, et al., 1982; Stein, et al., 1988, 1989; Levine and Taiwo, 1989) and reduce excitability of primary afferents (Sastry, 1978; Russell, et al., 1987). The mechanisms by which these effects occur are not known. Opioid receptors on central terminals of primary afferents are known to inhibit glutamate (Kangrga and Randic´, 1991; MacDonald and Nelson, 1978) and substance P (Mudge, et al., 1979; Hirota, et al., 1985) release in the spinal cord. Since the cell body is the most accessible portion of the primary afferent, electrophysiological recordings from the cell body have been used to correlate the opioid-induced decrease in neurotransmitter release with changes in ion conductances. Opioids reduce action potential duration of primary afferents (Werz and Macdonald, 1982; Shen and Crain, 1989) by either a direct inhibition of Ca^{+2} channels (Gross and Macdonald, 1987; Attali, et al., 1989; Schroeder, et al., 1991) or indirectly by opening K^+ channels (Werz and Macdonald, 1983). Thus, since opioid receptors are transported to both the central and peripheral terminals (Zarbin, et al., 1990; Hassan, et al., 1993), opioid agonists may modulate ion channels at either terminal.

One other major effector coupled to opioid receptors is adenylyl cyclase. Opioids applied to peripheral terminals of primary afferents reduce inflammatory pain through inhibition of adenylyl cyclase (Collier and Roy, 1974; Levine and Taiwo, 1989; Przewlocka, et al., 1991) and also inhibit axon-reflex mediated release of neurotransmitter in the periphery (Bartho´ and Szolcsanyi, 1981; Brodin, et al., 1983; Lembeck and Donnerer, 1985; Yaksh, 1988). The mechanism that mediates increased excitability during the inflammatory response, the axon-reflex and the mechanism by which opioids inhibit both processes are not understood. However, the ability of opioids to inhibit both the increased excitability and pain associated with inflammation (Ferreira and Nakamura, 1979; Ferreira, et al., 1982; Russell, et al., 1987; Stein, et al., 1988, 1989) led to the hypothesis that the actions of opioids on adenylyl cyclase and ion channels are linked in this system. We chose to study the possible opioid modulation of a cAMP-modulated channel, I_h. I_h was first

described as I_f in pacemaker neurons of the heart (Brown and DiFrancesco, 1980; Yanagihara and Irisawa, 1980; DiFrancesco, et al., 1986) and I_h in DRG neurons (Mayer and Westbrook, 1983). It is a nonselective cation current which is activated upon hyperpolarization. I_h is thought to be an important current underlying cardiac pacemaker activity (Brown and DiFrancesco, 1980; DiFrancesco, et al., 1988; Noble, et al., 1992), as well as being important in the modulation of firing characteristics in central neurons (Bobker and Williams, 1989; McCormick and Pape, 1990a; Pape, 1992). The purpose of this study was to determine the action of opioids on I_h in guinea pig primary afferent neurons of the nodose ganglion.

RESULTS

A subpopulation of nodose ganglion neurons have I_h

Whole-cell recordings were made from dissociated guinea pig nodose ganglion neurons after 3-7 days in culture. A slowly activating, voltage-dependent inward current was evoked in some neurons during hyperpolarizing voltage steps from a holding potential of -40 mV (Fig. 1). The current activated between -50 mV and -80 mV (estimated by eye; -62.7 +/- 1.8 mV), was insensitive to Ba^{+2} (200 μM) ions and was blocked by Cs^{+} (2 mM) ions (n = 5). These characteristics were indicative of I_h, as previously studied in detail in both the heart (Brown and DiFrancesco, 1980; Yanagihara and Irisawa, 1980; DiFrancesco, et al., 1986) and DRG neurons (Mayer and Westbrook, 1983). In general, we discovered that only medium to large neurons expressed an I_h current. This is consistent with observations of Tokimasa, et al. (1990) that many small-diameter primary afferent neurons do not express I_h.

I_h is modulated by cAMP

Forskolin, an activator of adenylyl cyclase, augmented I_h as illustrated in Fig. 2. A current / voltage (I/V) plot of the instantaneous current (current at the beginning of each step) and the steady-state current (current at the end of each step) showed that forskolin increased both at potentials more negative than -40 mV (Fig. 2b; n = 22). Forskolin

increased the current measured at -60 mV by 53 ± 5 % (22/22), and at the V1/2 (-80 mV) by 39 ± 5 % in 15/22 neurons. The forskolin-induced increase in inward tail current amplitudes became smaller and was observed less frequently with greater hyperpolarizations (Fig. 2 a, c) suggesting a shift in the voltage dependence of activation of I_h without a change in maximal conductance. A plot of the tail current amplitudes measured at -60 mV after steps to pre-potentials was used as a measure of the voltage dependence of activation of I_h (Fig. 2c). Activation curves from tail current data (see Fig. 5c) during control and superfusion of substances that increase cAMP were fit with the Boltzmann equation to estimate V1/2 and slope values (Table 1). The effects of cAMP analogs and PGE_2 (an inflammatory substance known to increase cAMP) on the activation of I_h are compared to the effects with forskolin in Table 1. All four substances significantly shift the I_h activation curve to the right ($p < 0.05$; student's paired t-test).

Dideoxyforskolin (10 μM), an analog of forskolin that does not activate adenylyl cyclase, had no effect on I_h in 8/8 neurons.

Forskolin-activated I_h is modulated by opioids

Opioids inhibited forskolin-stimulated I_h in 28 of 179 neurons. Superfusion of $[Met]^5$ enkephalin (ME - 1 μM), a non-selective μ- and ∂-agonist, or DAMGO (300 nM), a selective μ-agonist, had no effect on I_h in 7/7 neurons tested in control solutions. However, in the presence of forskolin (10 μM), the opioids inhibited a portion of the forskolin-mediated increase in I_h (Fig. 3). In another experiment, neurons were held at -60 mV (approximately Vrest for these neurons) and drugs were superfused. Forskolin (10 μM) elicited an inward current that was insensitive to $BaCl_2$, but was blocked by CsCl superfusion (data not shown). DAMGO inhibited the forskolin-induced inward current in a concentration-dependent manner. A dose-response curve with DAMGO (n = 6) estimated an EC50 of 45 nM, suggesting that the opioid inhibition resulted from activation of a μ-opioid receptor (Fig. 4). Figure 4 also shows that opioid inhibition of forskolin-stimulated I_h was antagonized by naloxone (1 μM) (n = 7).

Opioid application inhibited the forskolin-stimulated shift in activation of I_h in a subpopulation of nodose ganglion neurons (9/22; Fig. 5 a, b). One way that opioid-sensitive neurons were distinguished from others was the fact that I_h was evident at more depolarized potentials. Opioid-sensitive neurons had an I_h which was first evident at -57 ± 2 mV (n = 9) whereas I_h in insensitive neurons typically became evident at -65 ± 3 mV (n = 13; $p < .05$). In order to further quantify the modulation of I_h in nodose neurons, activation curves were plotted from the tail current amplitudes evoked at -60 mV after steps to the indicated pre-potentials. The currents were then normalized to the maximum current measured within each drug application (I/I_{max}) (Fig. 5c). Forskolin shifted the activation curves to the right by 11 ± 1 mV (measured at the V1/2). ME (1 μM), in the presence of forskolin, shifted the activation curve to the left by 6 ± 1 mV (n = 5; $p < 0.05$), and DAMGO (100 nM) shifted the activation curve to the left by 6 ± 1 mV (n = 4; $p < 0.05$). Since the inhibition by ME (1 μM) and DAMGO (100 nM) were similar in magnitude, the responses were pooled. Opioids decreased the forskolin-mediated inward current (-242 ± 70 pA, n =9) at -60 mV by 73 ± 10 % (see Fig. 4).

Opioids act through inhibition of adenylyl cyclase

In order to confirm that opioids were inhibiting the forskolin-mediated inward current via inhibition of adenylyl cyclase, cAMP analogs (8-bromo-cAMP (8-B-cAMP), dibutyryl-cAMP (db-cAMP), and chloro-phenyl-thio-cAMP (CPT-cAMP) (1 mM)) were perfused (Fig. 6). 8-B-cAMP and CPT-cAMP significantly increased I_h amplitude and caused a shift in the activation of I_h similar to forskolin. Forskolin (10 μM), CPT-cAMP, and 8-bromo-cAMP evoked significant increases in I_h amplitude over control: 364 ± 139 pA (n = 22), 211 ± 47 pA (n = 6), and 144 ± 54 pA (n = 8), respectively ($p < 0.05$). Dibutyryl-cAMP (1 mM) did not significantly increase the I_h tail current (33 ± 41 pA, n = 6). Increases in tail current amplitudes evoked by the cAMP analogs were blocked by superfusion with CsCl (2 mM). An example of the increase in I_h with CPT-cAMP (1 mM) and block by cesium is shown in Figure 6. In neurons that responded to ME (1 μM) in the presence of forskolin, ME had no effect in the presence of CPT-cAMP (1 mM, n = 3) or 8-B-cAMP (1 mM, n = 3). Since cAMP analogs bypass adenylyl cyclase, the lack of

opioid modulation of I_h indicates that opioids act via inhibition of adenylyl cyclase.

DISCUSSION

Opioid Modulation of I_h

We have described an interaction between opioid inhibition of adenylyl cyclase and consequent modulation of a voltage-dependent ion channel. Some guinea pig nodose ganglion neurons have a nonselective cation current that resembles I_f in the heart (Brown and DiFrancesco, 1980; Yanagihara and Irisawa, 1980; DiFrancesco, et al., 1986) and I_h in DRG neurons (Mayer and Westbrook, 1983) and sympathetic ganglion neurons (Tokimasa and Akasu, 1990). I_h is known to be modulated by cAMP. In the heart, stimulation of the sympathetic nervous system releases norepinephrine that stimulates ß-adrenergic receptors to activate adenylyl cyclase, and among other effects, shift the activation of I_f to more depolarized potentials (Brown, et al., 1979; DiFrancesco, et al., 1986; Hagiwara and Irisawa, 1989). The release of ACh from vagal stimulation acts on muscarinic receptors to shift the activation curve of I_f to more hyperpolarized potentials (DiFrancesco and Tromba, 1988a, 1988b; Chang and Cohen, 1992). I_h is also modulated through a cAMP mechanism in bull-frog sympathetic neurons (Tokimasa and Akasu, 1990; Akasu and Tokimasa, 1992). There are also several sites in the CNS where the modulation of I_h by the cAMP second messenger system have been investigated (Bobker and Williams, 1989; McCormick and Pape, 1990b; Pape, 1992). This study demonstrates that opioids can modulate I_h in a subpopulation of guinea pig nodose ganglion neurons via inhibition of adenylyl cyclase.

As in other cells, forskolin increased I_h amplitude and shifted the voltage dependence of activation to more depolarized potentials in nodose ganglion neurons. I_h was observed only in older cultures (3-7 days). Although space-clamp of the processes that develop in older cultures was a potential problem, the slow kinetics of I_h reduce the risk of artifact. In addition, the shift in kinetics caused by forskolin were consistent with other studies (Hagiwara and Irisawa, 1989; Tokimasa and Akasu, 1990; Chang and Cohen, 1992). Series resistance error due to the large currents is a second potential problem in spite of the

use of series resistance compensation (see Methods). Errors due to series resistance would result in a slower activation of I_h at negative potentials, such that our results may be an underestimate of the effects of forskolin.

Opioid modulation of I_h in the presence of forskolin only occurred in a subset of nodose ganglion neurons: medium-sized neurons with a low threshold for I_h. We have confirmed observations made by Tokimasa, et al. (1990) that many small-diameter primary afferent neurons do not express I_h. Opioids shifted I_h back to more hyperpolarized potentials in the presence of forskolin, but had no effect in the absence of forskolin. This is consistent with other studies showing that opioid inhibition of adenylyl cyclase is not evident in control at basal levels of cAMP (Collier and Roy, 1974; Przewlocka, et al., 1991). It is also consistent with electrophysiological studies showing that superfusion of opioids onto rabbit nodose (Shefner, et al., 1981) and spinal ganglion neurons (Williams and Zieglgansberger, 1981) had no effect on the resting membrane potential (-60 mV). On the basis of these previous studies, it was important to show that the modulation of I_h by opioids was due to a specific action on opioid receptors to inhibit adenylyl cyclase. Blockade of the inhibition by naloxone indicates that modulation of I_h is due to a specific action on opioid receptors. A recent study (Zarbin, et al., 1990) showed that 75% of vagal opioid receptors were the μ-subtype. The EC50 of DAMGO (45 nM) determined in this study is consistent with activation of μ-receptors in the locus coeruleus (Williams, et al., 1987), spinal trigeminal nucleus (Grudt and Williams, 1994), raphe magnus (Pan, et al., 1991) and μ-receptor inhibition of adenylate cyclase reported in human neuroblastoma SH-SY5Y cells (Yu, et al., 1990; Carter and Medzihradsky, 1992). The k-agonist, U69593, caused an inhibition of the forskolin modulation of I_h in 4/14 neurons (data not shown). This inhibition was small compared to the μ-agonist effects and were not studied further. DAMGO (100 nM) and ME (1 μM) had comparable responses in the same neurons suggesting that ME was acting through μ-opioid receptors. The effects of selective ∂-agonists have not been tested. Perfusion of cAMP analogs was used in order to directly augment I_h without stimulating adenylyl cyclase. Opioid inhibition of I_h in nodose neurons was not effective during superfusion of cAMP analogs in neurons confirmed to be

opioid-sensitive, suggesting that the modulation of I_h by opioids does occur via inhibition of adenylyl cyclase.

Many substances released in the inflammatory response increase cAMP production in neurons, including bradykinin, serotonin and PGE_2 (Suidan, et al., 1991; Zabala, et al., 1992; Collier and Roy, 1974). Serotonin augments I_h in cultured trigeminal ganglion neurons (Williams and Ingram, unpublished data) and can augment I_h through a cAMP pathway in central neurons (Bobker and Williams, 1989; McCormick and Pape, 1990b). Preliminary experiments with PGE_2 show that it shifts the activation curve of I_h in the depolarizing direction. PGE_2 and forskolin also depolarized nodose ganglion neurons during current-clamp experiments, an effect that was blocked by cesium in a proportion of cells (data not shown). Peripheral ganglia contain a heterogeneous population of cell types with different sensitivities to these inflammatory agents. We were most interested in finding a subpopulation of cells that was sensitive to opioids, so we chose to use forskolin in these experiments to reproducibly increase cAMP levels in all cells. It is now important to identify opioid-responsive neurons such that the physiological relevance of opioid modulation of I_h can be determined.

Functional significance

It has been shown that injections of opioid agonists into the periphery decrease experimental hyperalgesia (Ferreira and Nakamura, 1979; Ferreira, et al., 1982; Stein, et al., 1988, 1989; Levine and Taiwo, 1989). Opioid inhibition of inflammatory pain is associated with a decrease in cAMP (Ferreira and Nakamura, 1979; Beubler and Lembeck, 1980; Levine and Taiwo, 1989). Both hyperalgesia and inflammation are associated with increased neuronal excitability (Schaible and Schmidt, 1985; Kessler, et al., 1992; Birrell, et al., 1993; Rueff and Dray, 1993). Since inflammation and hyperalgesia are closely associated with an increase in cAMP, I_h in these neurons could be shifted to more positive potentials and potentially depolarize these peripheral neurons. This might explain the ability of inflammatory mediators to sensitize peripheral afferents and increase the sensation of pain. Local injections of opioids would act to decrease the intracellular levels of cAMP,

inhibit I_h and, thus, modulate the increased excitability of primary afferents due to inflammation and hyperalgesia.

EXPERIMENTAL PROCEDURES

CULTURE. Nodose ganglia were dissected from adult guinea pigs and washed. The culture technique was adopted from Beech, et al. (1991). Briefly, the ganglia were minced and dissociated for ten minutes in 2 ml of 20 units/ml papain (with 0.4 mg/ml cysteine) dissolved in Hanks balanced salt solution. They were placed into a second solution of dispase (25 u/ml) and collagenase (730 u/ml) for ten minutes. After dissociation, the enzymes were inhibited by washing twice with growth medium (10 mls MEM containing 10% FCS, 20 nM NGF, 100 u/ml pen-strep) and triturated three times through a 200 µl pipet tip. Neurons were suspended in 300 µl of growth medium and plated onto coverslips coated with 20 µl/ml polylysine and 40 µg/ml laminin. Media was replaced every two days. Recordings were generally made after three days to one week in culture.

RECORDINGS. Whole-cell recordings were made at 37°C with glass electrodes pulled and fire-polished to obtain a 4-6 MΩ pipette resistance. Both capacitance and series resistance compensation were used. Control medium contained in mM: NaCl 126; $MgCl_2$ 1.2; KCl 5; $CaCl_2$ 2.5; HEPES 10; and dextrose 30 mM, pH 7.28. The control internal pipette solution consisted of KGluconate 128; KCl 10; $MgCl_2$ 1; HEPES 10; EGTA 1; free Ca^{+2} 30 nM; Na_2ATP 2; NaGTP 0.25. Drugs used were forskolin, dideoxyforskolin, 8-bromo-cAMP, dibutyryl-cAMP, chloro-phenyl-thio-cAMP, PGE_2, $[Met]^5$ enkephalin, and DAMGO (SIGMA Chemical Co., St. Louis, MO). They were dissolved into the external solution and applied via gravity superfusion. Unless otherwise indicated, experiments were done in the presence of $BaCl_2$ (200 µM) to decrease the involvement of K^+ currents when neurons were held at positive holding potentials.

DATA ANALYSIS. Data was collected via an Axopatch 1-D amplifier and filtered at 2

kHz. Currents were digitized and recorded with Pclamp software and analyzed using Axograph (Axon Instruments, Inc.) and Kaleidograph software. Concentration-response and activation curves were derived using a Levenberg-Marquardt algorithm to fit a logistic function (to estimate EC50) and a Boltzmann function (to estimate V1/2 and slope values) respectively (Kaleidograph, Synergy Software). Statistical significance of voltage shifts was determined with repeated measures t-tests. A p value of less than 0.05 was taken as indicating statistical significance. Descriptive statistics used were the mean and standard error of the mean.

REFERENCES

Akasu, T., and Tokimasa, T. (1992). Cellular metabolism regulating H and M currents in bullfrog sympathetic ganglia. Can. J. Physiol. Pharmacol. *70*, S51-S55.

Attali, B., Saya, D., Nah, S., and Vogel, Z. (1989). *k*-opiate agonists inhibit Ca^{+2} influx in rat spinal cord-dorsal root ganglion cocultures. J. Biol. Chem. *264*, 347-353.

Bartho´, L., and Szolcsanyi, J. (1981). Opiate agonists inhibit neurogenic plasma extravasation in the rat. Eur. J. Pharmacol. *73*, 101-104.

Beech, D. J., Bernheim, L., Mathie, A., and Hille, B. (1991). Intracellular Ca^{+2} buffers disrupt muscarinic suppression of Ca^{+2} current and M current in rat sympathetic neurons. Proc. Natl. Acad. Sci. USA *88*, 652-656.

Beubler, E., and Lembeck, F. (1980). Inhibition by morphine of prostaglandin E_1-stimulated secretion and cyclic adenosine 3',5'-monophosphate formation in the rat jejunum in vivo. Br. J. Pharmacol. *68*, 513-518.

Birrell, G. J., McQueen, D. S., Iggo, A., and Grubb, B. D. (1993). Prostanoid-induced potentiation of the excitatory and sensitizing effects of bradykinin on articular mechanonociceptors in the rat ankle joint. Neuroscience *54*, 537- 544.

Bobker, D. H., and Williams, J. T. (1989). Serotonin augments the cationic current Ih in central neurons. Neuron *2*, 1535-1540.

Brodin, E., Gazelius, B., Panopoulos, P., and Olgart, L. (1983). Morphine inhibits substance P release from peripheral sensory nerve endings. Acta Physiol. Scand. *117*, 567-570.

Brown, H. F., DiFrancesco, D., Noble, S. J. (1979). How does adrenalin accelerate the heart? Nature *280*, 235-236.

Brown, H., and DiFrancesco, D. (1980). Voltage-clamp investigations of membrane currents underlying pace-maker activity in rabbit sino-atrial node. J. Physiol. *308*, 331-351.

Carter, B. D., and Medzihradsky, F. (1992). Opioid signal transduction in intact and fragmented SH-SY5Y neural cells. J. Neurochem. *58*, 1611-1619.

Chang, F., and Cohen, I. S. (1992). Mechanism of acetylcholine action on pacemaker current (i_f) in canine Purkinje fibers. Pflugers Arch. *420*, 389-392.

Christie, M. J., Williams, J. T., and North, R. A. (1987). Cellular mechanisms of opioid tolerance: studies in single brain neurons. Mol. Pharm. *32*, 633-638.

Collier, H. O. J., and Roy, A. C. (1974). Inhibition of E prostaglandin-sensitive adenyl cyclase as the mechanism of morphine analgesia. Prostaglandins *7*, 361-375.

DiFrancesco, D., Ferroni, A., Mazzanti, M., and Tromba, C. (1986). Properties of the hyperpolarizing-activated current *(i_f)* in cells isolated from the rabbit sino-atrial node. J. Physiol. *377*, 61-88.

DiFrancesco, D., and Tromba, C. (1988a). Inhibition of the hyperpolarization-activated current (i_f) induced by acetylcholine in rabbit sino-atrial node myocytes. J. Physiol. *405*, 477-491.

DiFrancesco, D., and Tromba, C. (1988b). Muscarinic control of the hyperpolarization-activated current (i_f) in rabbit sino-atrial node myocytes. J. Physiol. *405*, 493-510.

DiFrancesco, D., Ducouret, P., Robinson, R. B. (1989). Muscarinic modulation of cardiac rate at low acetylcholine concentrations. Science *243*, 669-671.

Ferreira, S. H., and Nakamura, M. (1979). Prostaglandin hyperalgesia: the peripheral analgesic activity of morphine, enkephalins and opioid antagonists. Prostaglandins *18*, 191-200.

Ferreira, S. H., Molina, N., and Vettore, O. (1982). Prostaglandin hyperalgesia, V: a peripheral analgesic receptor for opiates. Prostaglandins *23*, 53-60.

Gross, R. A., and Macdonald, R. L. (1987). Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture. Proc. Natl. Acad. Sci. USA *84*, 5469-5473.

Gross, R. A., Moises, H. C., Uhler, M. D., and Macdonald, R. L. (1990). Dynorphin A and cAMP-dependent protein kinase independently regulate neuronal calcium currents. Proc. Natl. Acad. Sci. USA *87*, 7025-7029.

Grudt, T. J., and Williams, J. T. (1994). μ-opioid agonists inhibit spinal trigeminal substantia gelatinosa neurons in guinea pig and rat. J. Neuroscience, in press.

Hagiwara, N., and Irisawa, H. (1989). Modulation by intracellular Ca^{+2} of the hyperpolarization-activated inward current in rabbit single sino-atrial node cells. J. Physiol. *409*, 121-141.

Hassan, A. H. S., Ableitner, A., Stein, C., and Herz, A. (1993). Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. Neuroscience *55*, 185-195.

Hirota, N., Kuraishi, Y., Hino, Y., Sato, Y., Satoh, M., and Takagi, H. (1985). Met-enkephalin and morphine but not dynorphin inhibit noxious stimuli-induced release of substance P from rabbit dorsal horn in situ. Neuropharmacology *24*, 567-570.

Kangrga, I., and Randic´, M. (1991). Outflow of endogenous aspartate and glutamate from the rat spinal dorsal horn in vitro by activation of low- and high-threshold primary afferent fibers. Modulation by μ-opioids. Brain Research *553*, 347-352.

Kessler, W., Kirchhoff, C., Reeh, P. W., and Handwerker, H. O. (1992). Excitation of cutaneous afferent nerve endings *in vitro* by a combination of inflammatory mediators and conditioning effect of substance P. Exp. Brain Res. *91*, 467-476.

Lembeck, F., and Donnerer, J. (1985). Opioid control of the function of primary afferent substance P fibres. Eur. J. Pharmacol. *114*, 241-246.

Levine, J. D., and Taiwo, Y. O. (1989). Involvement of the mu-opiate receptor in peripheral analgesia. Neuroscience *32*, 571-575.

Macdonald, R. L., and Nelson, P. G. (1978). Specific-opiate-induced depression of transmitter release from dorsal root ganglion cells in culture. Science *199*, 1449-1450.

Mayer, M. L., and Westbrook, G. L. (1983). A voltage-clamp analysis of inward (anomalous) rectification in mouse spinal sensory ganglion neurones. J. Physiol. *340*, 19-45.

McCormick, D. A., and Pape, H-C. (1990a). Properties of a hyperpolarization-activated cation current and its role in rhythmic oscillation in thalamic relay neurones. J. Physiol. *431*, 291-318.

McCormick, D. A., and Pape, H-C. (1990b). Noradrenergic and serotonergic modulation

of a hyperpolarization-activated cation current in thalamic relay neurones. J. Physiol. *431*, 319-342.

Mudge, A. W., Leeman, S. E., and Fischbach, G. D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. Proc. Natl. Acad. Sci. USA *76*, 526-530.

Noble, D., Denyer, J. C., Brown, H. F., and DiFrancesco, D. (1992). Reciprocal role of the inward currents $i_{b,Na}$ and i_f in controlling and stabilizing pacemaker frequency of rabbit sino-atrial node cells. Proc. R. Soc. Lond. Biol. *250*, 199-207.

Pan, Z. Z., Williams, J. T., and Osborne, P. B. (1990). Opioid actions on single nucleus raphe magnus neurons from rat and guinea-pig in vitro. J. Physiol. *427*, 519-532.

Pape, H-C. (1992). Adenosine promotes burst activity in guinea-pig geniculocortical neurones through two different ionic mechanisms. J. Physiol. *447*, 729-753.

Przewlocka, B., Dziedzicka, M., Lason, W., and Przewlocki, R. (1991). Differential effects of opioid receptor agonists on nociception and cAMP level in the spinal cord of monoarthritic rats. Life Sci. *50*, 45-54.

Rueff, A., and Dray, A. (1993). Sensitization of peripheral afferent fibres in the *in vitro* neonatal rat spinal cord-tail by bradykinin and prostaglandins. Neuroscience *54*, 527-535.

Russell, N. J. W., Schaible, H. G., and Schmidt, R. F. (1987). Opiates inhibit the discharges of fine afferent units from inflamed knee joint of the cat. Neurosci. Lett. *76*, 107-112.

Sastry, B. R. (1978). Morphine and met-enkephalin effects on sural A∂ afferent terminal excitability. Eur. J. Pharmacol. *50*, 269-273.

Schaible, H. G., and Schmidt, R. F. (1985). Effects of an experimental arthritis on the sensory properties of fine articular afferent units. J. Neurophys. *54*, 1109-1122.

Schroeder, J. E., Fischbach, P. S., Zheng, D., and McCleskey, E. W. (1991). Activation of μ opioid receptors inhibits transient high- and low-threshold Ca^{+2} currents, but spares a sustained current. Neuron *6*, 13-20.

Shefner, S. A., North, R. A., and Zukin, R. S. (1981). Opiate effects on rabbit vagus nerve: electrophysiology and radioligand binding. Brain Research *221*, 109-116.

Shen, K. -F., and Crain, S. M. (1989). Dual opioid modulation of the action potential duration of mouse dorsal root ganglion neurons in culture. Brain Research *491*, 227-242.

Stein, C., Millan, M. J., Shippenberg, T. S., and Herz, A. (1988). Peripheral effect of fentanyl upon nociception in inflamed tissue of the rat. Neurosci. Lett. *84*, 225-228.

Stein, C., Millan, M., Shippenberg, T. S., Peter, K., and Herz, A. (1989). Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta, and kappa receptors. J. Pharmacol. Exp. Ther. *248*, 1269-1275.

Suidan, H. S., Murrell, R. D., and Tolkovsky, A. M. (1991). Carbachol and bradykinin elevate cyclic AMP and rapidly deplete ATP in cultured rat sympathetic neurons. Cell Regul. 2, 13-25.

Tokimasa, T., and Akasu, T. (1990). Cyclic AMP regulates an inward rectifying sodium-potassium current in dissociated bull-frog sympathetic neurones. J. Physiol. *420*, 409-429.

Tokimasa, T., Shiraishi, M., and Akasu, T. (1990). Morphological and

electrophysiological properties of C-cells in bullfrog dorsal root ganglia. Neurosci. Lett. *116*, 304-308.

Werz, M. A., and Macdonald, R. L. (1982). Heterogeneous sensitivity of cultured dorsal root ganglion neurones to opioid peptides selective for μ- and ∂-opiate receptors. Nature *299*, 730-733.

Werz, M. A., and Macdonald, R. L. (1983). Opioid peptides selective for mu- and delta-opiate receptors reduce calcium-dependent action potential duration by increasing potassium conductance. Neurosci. Lett. *42*, 173-178.

Williams, J. T., and Zieglgansberger, W. (1981). Mature spinal ganglion cells are not sensitive to opiate receptor mediated actions. Neurosci. Lett. *21*, 211-216.

Yaksh, T. L. (1988). Substance P release from knee joint afferent terminals: modulation by opioids. Brain Research *458*, 319-324.

Yanagihara, K., and Irisawa, H. (1980). Inward current activated during hyperpolarization in the rabbit sinoatrial node cell. Pflugers Arch. *385*, 11-19.

Yu, V. C., Eiger, S., Duan, D., Lameh, J., and Sadee, W. (1990). Regulation of cyclic AMP by the μ-opioid receptor in human neuroblastoma SH-SY5Y cells. J. Neurochem. *55*, 1390, 1396.

Zabala, M. T., Lorenzo, P., Alvarez, L., Berlanga, J. J., and Garc'ia-Ru'iz, J. P. (1992). Serotonin increases the cAMP concentration and the phosphoenolpyruvate carboxykinase mRNA in rat kidney, small intestine, and liver. J. Cell. Physiol. *150*, 451-455.

Zarbin, M. A., Wamsley, J. K., and Kuhar, M. J. (1990). Anterograde transport of opioid receptors in rat vagus nerves and dorsal roots of spinal nerves: pharmacology and

sensitivity to sodium and guanine nucleotides. Exp. Brain Res. *81*, 267-278.

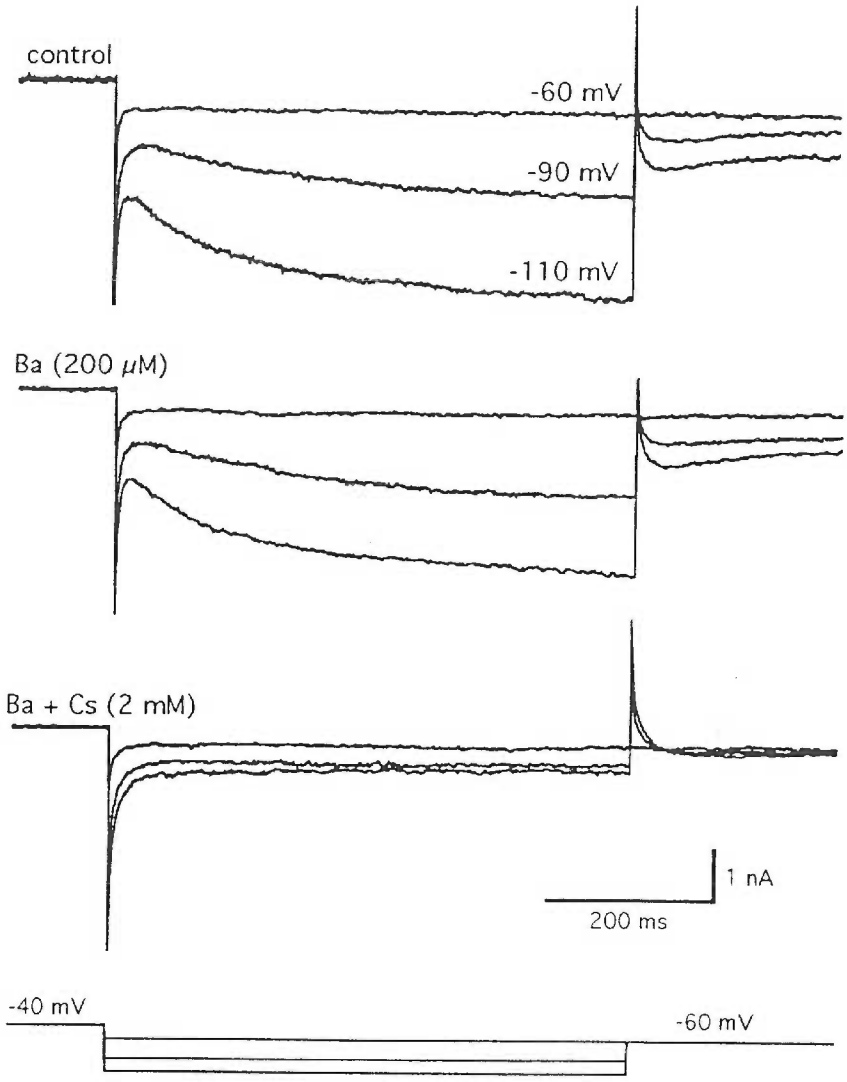

Figure 1. I_h characteristics.

I_h currents are elicited by hyperpolarizing steps from -40 mV to -60, -90, and -110 mV in control, Ba^{+2} (200 μM) and Cs^+ (2 mM) as indicated.

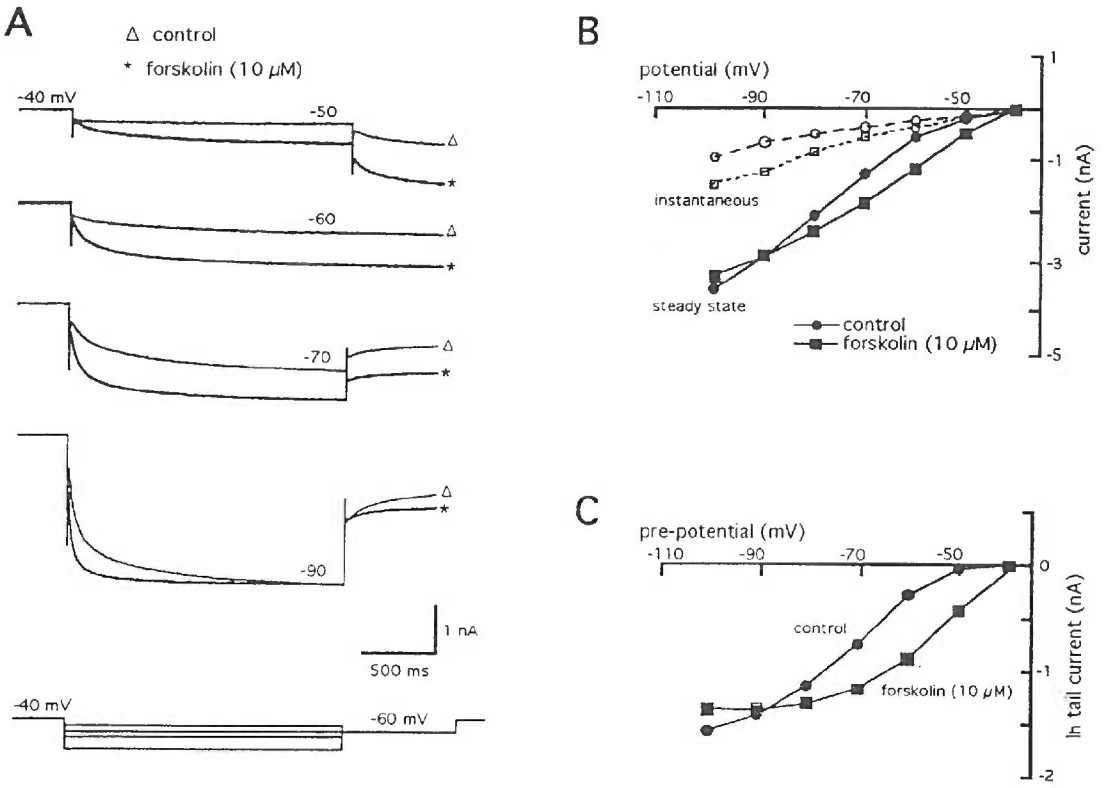

Figure 2. cAMP-dependent modulation of I_h.

(A) I_h was elicited by hyperpolarization protocol (shown below) with a return step to -60 mV to measure tail currents. Selected traces are shown in control (Δ) and in forskolin (10 μM) (*). (B) A current-voltage plot of the the effects of forskolin on instantaneous (open symbols) and steady-state (closed symbols) current. Instantaneous measurements were taken at the beginning of the hyperpolarizing step, while steady-state measurements were taken at the end of the hyperpolarizing step. (C) A current-voltage plot of I_h tails measured at -60 mV in the presence and absence of forskolin.

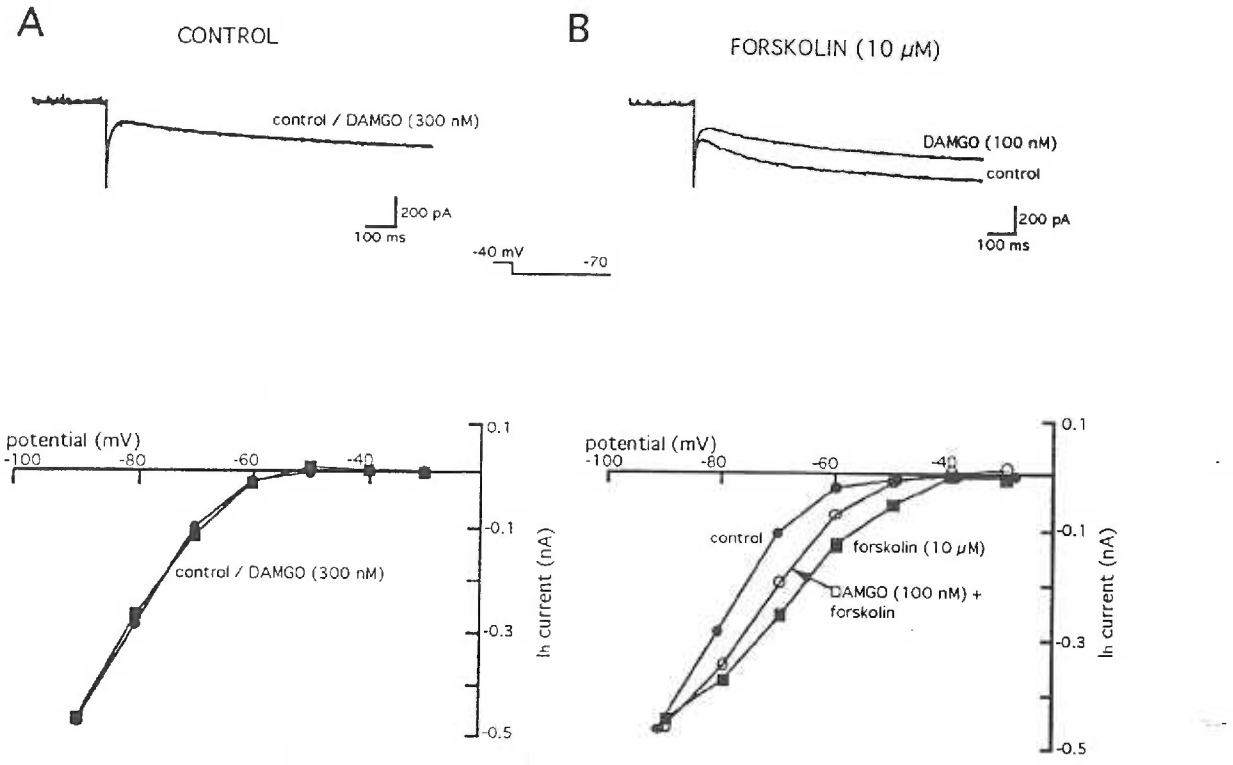

Figure 3. Opioids only modulate I_h in the presence of forskolin. Current/voltage plots of steady-state current (measured at end of the step) minus instantaneous current (measured at the beginning of the step) in control (A), and in forskolin (10 μm) (B). Representative traces elicited by stepping to -70 mV from a holding potential of -40 mV are shown above. DAMGO only inhibits I_h in the presence of forskolin.

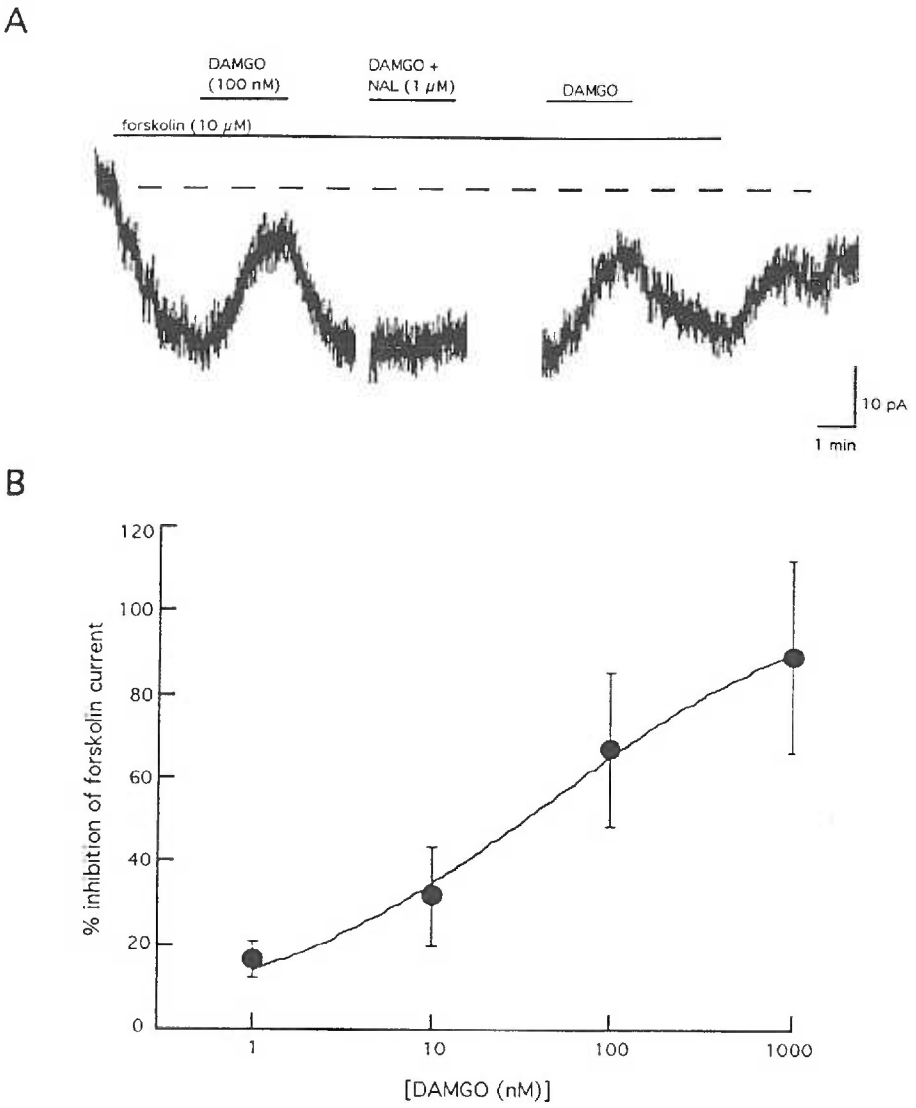

Figure 4 . Opioid inhibition is dose-dependent.

(A) Current recording from a cell held at -60 mV (near the resting membrane potential). Drug superfusion is indicated by the solid lines. The dashed line represents the baseline current from the beginning of the experiment. Forskolin (10 μM) caused an inward current that was reversed by DAMGO (100 nM). DAMGO had no effect in the presence of naloxone (1 μM) but the DAMGO effect recovered after washout of naloxone. Gaps in the trace reflect other drug applications and washout periods. (B) A dose-response curve was generated from data of 6 neurons receiving four concentrations of DAMGO (1nM, 10 nM, 100 nM, 1 μM) as in the protocol described in (A). Large error bars reflect the variability between cells. A Levenberg-Marquardt algorithm curve fit (Kaleidograph, Synergy software) estimated an EC50 = 45 ± 38 nM.

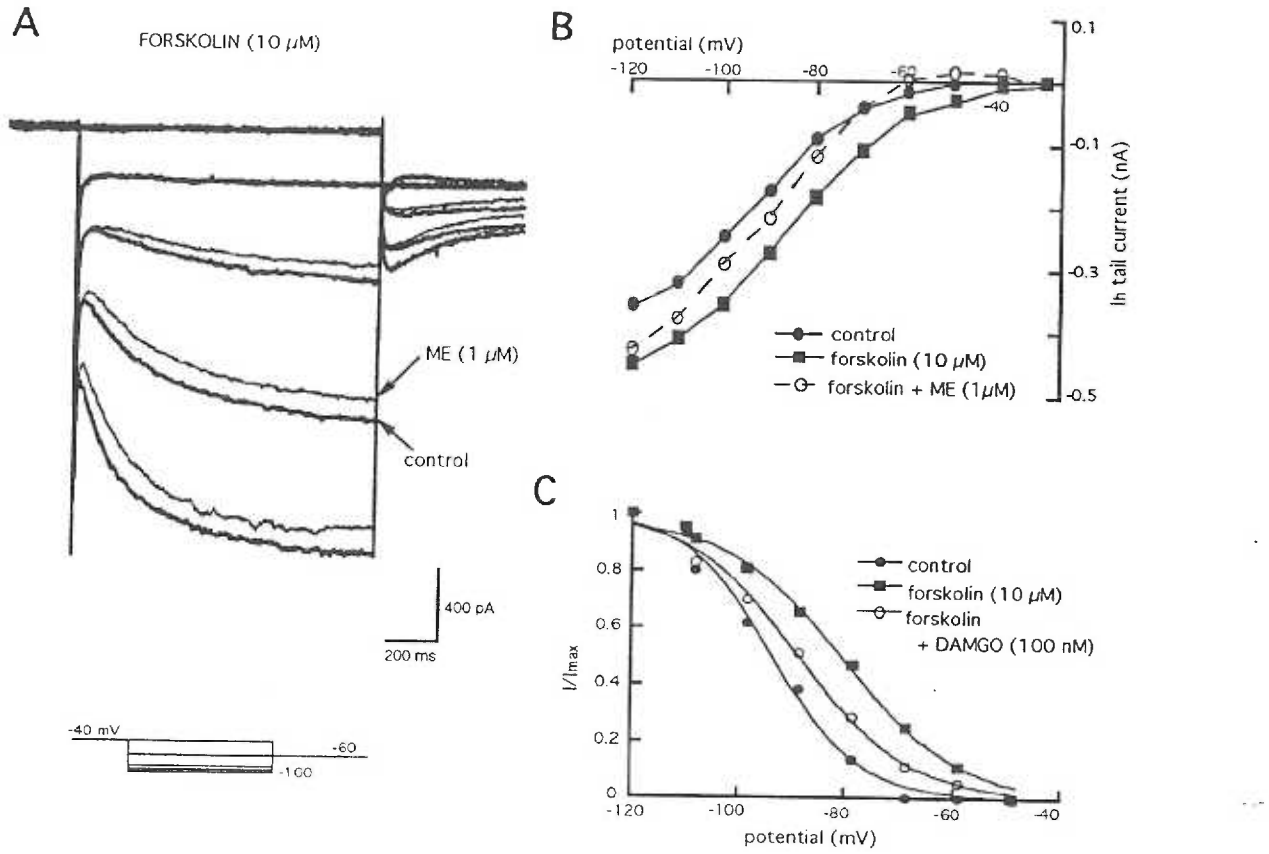

Figure 5. Opioids inhibit forskolin-stimulation of I_h.

I_h currents elicited by hyperpolarizing steps from a holding potential of -40 mV (protocol shown below) in the presence of forskolin (10 μM). (A) Selected traces show the inhibition of I_h in the presence of forskolin by ME (1 μM). (B) Current/voltage plot of tail current amplitudes shown in (A). (C) In another neuron, activation curves were generated from tail current amplitudes normalized to the maximum tail current amplitude for each drug application (I/Imax). Activation curves were fitted with the Boltzmann equation (Kaleidograph, Synergy Software) to estimate V1/2 and slope values.

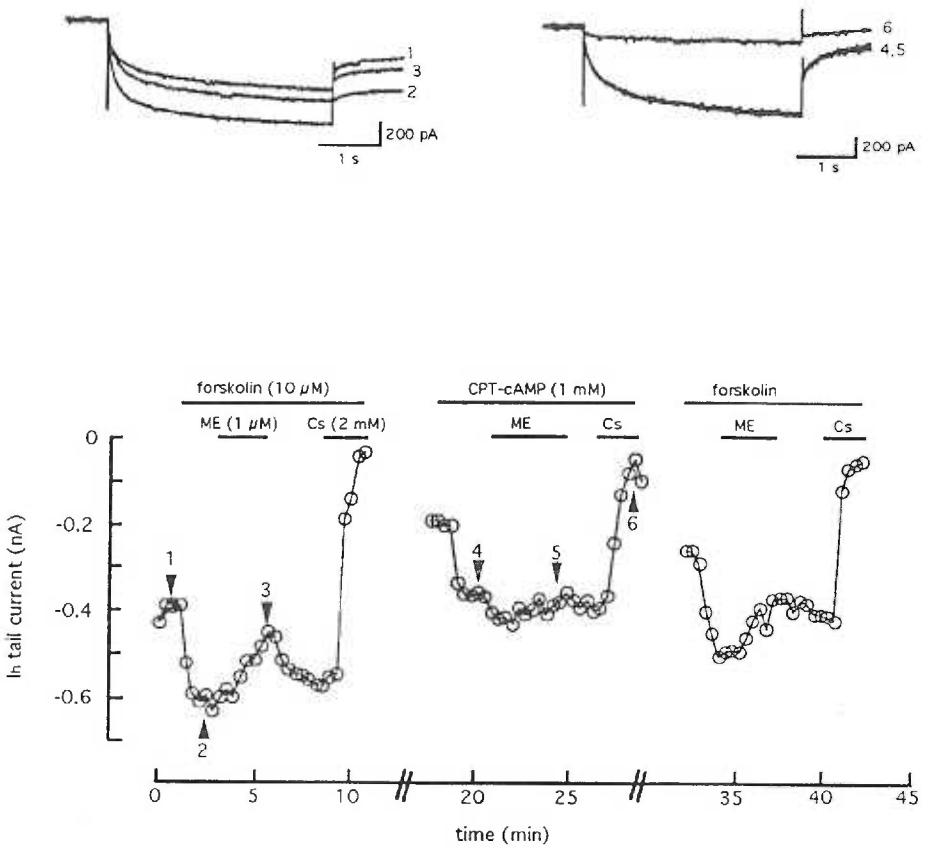

Figure 6. Opioids are ineffective when I_h is stimulated by cAMP analogs. A nodose ganglion neuron was held at -40 mV and stepped to -70 mV every 20 s. Tail current amplitudes measured at the return step to -60 mV provided the measure of I_h. Drug applications are shown by bars. Representative traces designated by the numbers are shown above. Forskolin increases the inward current which can be inhibited reversibly by ME (1 μM). Both basal I_h and the forskolin-mediated increase in I_h are blocked by Cs (2 mM). ME has no effect when I_h is stimulated by CPT-cAMP (1 mM), but this increase in I_h is also blocked by cesium. Breaks in the time axis represent the long washout periods of forskolin and CPT-cAMP respectively.

Table 1.

	n	V1/2 control	V1/2 drug	slope control	slope drug
forskolin (10 μM)	9	-79 ± 2 mV	-68 ± 2 mV *	8.8 ± 0.8	10.7 ± 0.6*
CPT-cAMP (1 μM)	5	-70 ± 1 mV	-63 ± 1 mV*	6.9 ± 0.5	5.7 ± 0.3 NS
8-bromo-cAMP (1 μM)	5	-73 ± 2 mV	-67 ± 1 mV*	6.6 ± 0.5	7.8 ± 0.6 NS
PGE2 (1 μM)	3	-71 ± 5 mV	-64 ± 5 mV*	6.4 ± 0.6	7.3 ± 0.6

*__Table 1.__ Forskolin, cAMP analogs, and PGE2 shift the I_h activation curves. V1/2 and slope values estimated from Boltzmann fits of I_h activation curves are compared between control and drug applications. Significance was tested with repeated measures t-tests, *signifies $p < 0.05$.

Neuron

Information for Contributors (1995)

Scope of Articles

Neuron publishes reports of novel results in any area of experimental neuroscience. Papers will be considered for publication if they report results of unusual significance or general interest. Papers should be as concise as possible. The principal text of manuscripts (Summary through Discussion) is usually no more than 17 double-spaced pages; there should be fewer than 8 figures. In terms of a file on disk, the limit corresponds to a total text length of about 60K, corresponding usually to about 35K of principal text with about 25K of all other text. All of the figures should fit into no more than 2 pages.

Submission of Research Articles

Authors should submit to the *Neuron* office in Cambridge. **Four** copies of each manuscript should be provided. If a manuscript is closely related to papers in press or submitted elsewhere, it will be considered for publication only after copies of these papers are provided. Under no circumstances will any paper be considered that contains any data that have been or will be submitted for publication elsewhere (including symposium volumes).

Organization and General Style of Research Articles

Summary

A single paragraph of fewer than 120 words.

Running Title

No more than 50 characters.

Introduction

Succinct, with no subheadings.

Results and Discussion

These sections may each be divided by subheadings or may be combined. *Footnotes should not be used and will be transferred to the text.*

Genetic loci should be italicized; protein products of the loci are not italicized, nor are journal names, foreign phrases, or species names. Units should conform with the International System of Units (SI). If special circumstances necessitate the use of other units, these should be defined on first appearance in terms of SI units. Nonstandard abbreviations should be defined when first used in the text. All text should be letter quality and double spaced.

Experimental Procedures

This section should contain sufficient detail so that all procedures can be repeated, in conjunction with cited references.

References

The references include only articles that are published or in press. Unpublished data, submitted manuscripts, or personal communications should be cited within the text. Personal communication should be documented by a letter of permission. Abstracts of work presented at meetings may not be cited.

Please use the following style:

Miller, C. (1989). Genetic manipulation of ion channels: a new approach to structure and mechanism. Neuron 2, 1195-1205.

Fallon, J. H., and Loughlin, S. E. (1993). Functional implications of the anatomical localization of neurotrophic factors. In Neurotrophic Factors, S. E. Loughlin and J. H. Fallon, eds. (San Diego, California: Academic Press), pp. 1-24.

Figures

Each copy of a submitted manuscript should be provided with a set of figures of sufficient quality for reviewers to judge the data. Magnification should be indicated by a bar scale. Lettering on halftone figures generated by computer should be outside the area of the halftone. Color figures will be included only when in the reviewers' opinions they are essential. Authors will be charged $1500 for the first page, $1000 for the second, and $250 for each additional page, which partially defrays the cost of separating and printing color figures.

Figure Legends and Tables

Should be included in the submitted manuscript as separate sections, following the style of the journal. Tables must be double spaced.

Publication Schedule

Papers that do not conform to the general criteria for publication will be returned immediately, without detailed review. Otherwise, we try to let authors have reviewers' comments in less than a month. If revisions are a condition of publication, only one revised version of the paper will be considered and must be submitted within 3 months of the initial decision. Articles will be published within 12 weeks of acceptance.

Typesetting from Disk

All authors must provide a disk copy of each manuscript. Disks should preferably be formatted under MS-DOS (PC-DOS); however, we can handle 3.5" disks from other MS-DOS and Macintosh operating systems. Please indicate the word processor program, the type of computer, and the operating system used to generate the file.

Distribution of Material

Publication of a research article in *Neuron* is taken to imply that the authors are prepared to distribute freely to academic researchers for their own use any materials (e.g., cells, DNA, antibodies, genetically engineered mice) used in the published experiments. In cases of dispute, authors may be required to make primary data available to the Editor. Nucleic acid and protein sequences as well as X-ray crystallographic coordinates should be deposited in the appropriate data base.

Minireviews

A minireview discusses briefly a sharply focused topic of recent experimental research and makes it accessible to researchers in other areas. Therefore, it should provide a critical but balanced view of the field. Proposals for minireviews should be submitted to the Editor.

Manuscript 2:

Journal of Physiology, submitted.

Modulation of I_h by cyclic-nucleotides in guinea pig primary afferent neurons

Susan L. Ingram and John T. Williams*

from

The Vollum Institute, OHSU, Portland, OR 97201, U.S.A.

*Corresponding author:
Vollum Institute, L474
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road
Portland OR 97201
Tel (503) 494-5465
FAX (503) 494-6972
Email williamj@OHSU.EDU

Abbreviated title: I_h in primary afferents

Key words: ion channels, cyclic adenosine monophosphate (cAMP), prostaglandin E

SUMMARY

1. Whole-cell patch-clamp recordings were made from dissociated guinea-pig nodose and trigeminal ganglion neurons in culture to study second messenger mechanisms of I_h modulation.

2. Prostaglandin E2 (PGE2; 1 μM) and forskolin (10 μM) modulate I_h in primary afferents by shifting the activation curve in the depolarizing direction and increasing the maximum amplitude.

3. The cAMP analogs, RP-cAMP-S (an inhibitor of PKA) and SP-cAMP-S (an activator of PKA), both shifted the activation curve of I_h to more depolarized potentials and occluded the effects of forskolin. These results suggest that I_h is modulated by a direct action of the cAMP analogs.

4. Superfusion of other cyclic nucleotide analogs (8Br-cAMP, CPT-cAMP, 8Br-cGMP (1 mM)) mimicked the actions of forskolin and PGE2, but db-cGMP (1 mM), 5' AMP (1 mM) and adenosine (1 mM) had no effect on I_h. 8Br-cAMP and 8Br-cGMP had similar concentration response profiles suggesting that I_h has little nucleotide selectivity.

5. The inhibitor peptide (PKI; 20 μM), the catalytic subunit of PKA (C subunit; 1.5 μM) and phosphatase inhibitors (microcystin (2 μM) and okadaic acid (1 μM)) had no effect on forskolin modulation of I_h.

6. These results indicate that I_h is regulated by cyclic-nucleotides in sensory neurons. Positive regulation of I_h by prostaglandins produced during inflammation may lead to depolarization and facilitation of repetitive activity, and thus, contribute to sensitization to painful stimuli.

INTRODUCTION

I_h is a hyperpolarization-activated nonselective cation current that has been described in the pacemaker cells of the heart (Yanagihara & Irisawa, 1980; DiFrancesco, Ferroni, Mazzanti & Tromba, 1986), in smooth muscle (Benham, Bolton, Denbigh & Lang, 1987), and in neurons (Mayer & Westbrook, 1983; Tokimasa & Akasu, 1990; Kamondi & Reiner, 1991). I_h plays a role in the generation of spontaneous action potentials (Brown & DiFrancesco, 1980; McCormick & Pape, 1990a; DiFrancesco, 1991; Noble, Denyer, Brown & DiFrancesco, 1992), and modulation of I_h results in the regulation of firing frequencies (DiFrancesco, Ducouret &Robinson, 1989; Denyer & Brown, 1990; McCormick & Pape, 1990b; Banks, Pearce & Smith, 1993). Activation of adenylyl cyclase in the heart causes a shift of the voltage dependence of I_h to more depolarized potentials (DiFrancesco, et al., 1986), whereas inhibition of adenylyl cyclase shifts the voltage dependence to more hyperpolarized potentials (DiFrancesco & Tromba, 1988; Chang & Cohen, 1992). The second messenger pathway leading to modulation of I_h involves regulation of adenylyl cyclase but the mechanism is not completely understood. Protein kinase inhibitors shifted I_h activation to more hyperpolarized potentials and blocked the effects of adenylyl cyclase activation in Purkinje cells of the heart (Chang, Cohen, DiFrancesco, Rosen & Tromba, 1991) and sympathetic neurons (Tokimasa & Akasu, 1990) implicating cAMP-dependent protein kinase A (PKA) in tonic and receptor-mediated regulation of I_h. However, other second messenger mechanisms have also been proposed. Activated G protein α-subunits (Gs, Go, and Gi) mimicked the effects of noradrenaline and acetylcholine when applied to inside-out patches from SA node cells suggesting that modulation may occur through a direct action of G proteins (Yatani, Okabe, Codina, Birnbaumer & Brown, 1990). Alternatively, direct application of cAMP and cAMP analogs augmented I_h in SA node myocytes (DiFrancesco & Tortora, 1991) and increased the probability of opening in single-channel recordings of I_h (DiFrancesco & Mangoni, 1994).

Prostaglandins are substances produced by the inflammatory cascade that produce hyperalgesia (Taiwo, Bjerknes, Goetzl & Levine, 1989). PGE2 increases cAMP levels in

cultured primary afferent neurons (Hingtgen, Waite & Vasko, 1995) and has also been shown to produce hyperalgesia and increase excitability of primary afferent neurons via a cAMP-dependent mechanism (Taiwo & Levine, 1991; Cui & Nicol, 1995). Since primary afferents have been shown to express I_h (Mayer & Westbrook, 1983), regulation of I_h by cAMP may play an important role in PGE2-induced excitation of primary afferents. The purpose of this study was to examine the effects of PGE2 on I_h and determine the mechanism by which cAMP modulates I_h in primary afferent neurons.

METHODS

Culture. Adult guinea pigs were anesthesized with halothane and killed by severing major blood vessels in the chest. Nodose and trigeminal ganglia were dissected and washed. The culture technique was adopted from Beech, Bernheim, Mathie & Hille (1991). Briefly, the ganglia were minced and dissociated for ten minutes in 2 ml of 20 units/ml papain (with 0.4 mg/ml cysteine) dissolved in Hanks balanced salt solution (no divalent cations). They were placed into a second solution of dispase (25 mg/ml) and collagenase (730 u/ml) for 25 minutes. After dissociation, the enzymes were inhibited by washing twice with growth medium (10 mls MEM containing 10% FCS, 50 ng/ml NGF, 100 u/ml pen-strep) and triturated through a glass pipette flamed to approximately 300 μm. Neurons were plated onto coverslips coated with 0.1 mg/ml polylysine and 40 μg/ml laminin, and the medium was replaced every two days. Recordings were made between 2-7 days after plating.

Recordings. Whole-cell recordings were made at 37°C with glass electrodes pulled and fire-polished to obtain a 2-4 MΩ pipette resistance. Access resistances ranged from 3-6 MΩ and the recordings could often be maintained for approximately 60 minutes. Both capacitance (10 - 40 pF) and series resistance (2 - 5 MΩ; 80%) compensation were used. Control medium contained in mM: NaCl 146; $MgCl_2$ 1.2; KCl 5; $CaCl_2$ 2.5; HEPES 5; and dextrose 30 mM, pH 7.3. The control internal pipette solution contained in mM: CsGluconate 140; NaCl 10; HEPES 10; EGTA 1; free Ca^{2+} 30 nM; Na_2ATP 2; NaGTP 0.25. Ca^{2+} experiments were done with internal solutions buffered with BAPTA (20

mM). $CaCl_2$ (17 μM) and $CaCl_2$ (9.1 mM) were added to the BAPTA internal solution to obtain a pCa10 and pCa7, respectively. Forskolin, PGE2 (SIGMA Chemical Co., St. Louis, MO), okadaic acid (Biomol, Plymouth Meeting, PA), and microcystin-LR (GIBCO BRL, Gaithersburg, MD) were dissolved into dimethyl sulfoxide stock solutions. Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (RP-cAMP-S), adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (SP-cAMP-S), 8-bromo-adenosine-3',5'-cyclic monophosphate (8Br-cAMP), and 8-bromo-guanosine-3',5'-cyclic monophosphate (8Br-cGMP) (BioLog, LaJolla, CA) were dissolved in water. 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (CPT-cAMP), N^2,2'-O-dibutyrylguanosine 3',5'-cyclic monophosphate (db-cGMP), adenosine 5'-monophosphate (5'AMP) (SIGMA Chemical Co., St. Louis, MO), and adenosine (Boerhinger-Mannheim, Indianapolis, IN) were dissolved directly into the extracellular solution to the final concentrations. The PKA inhibitor peptide fragment (5-24) (PKI) and PKA catalytic subunit (C subunit) were generous gifts from the laboratory of John Scott, Vollum Institute, Portland, OR. RP-cAMP-S, SP-cAMP-S, PKI, C subunit, okadaic acid and microcystin were added to the internal pipette solution while all other substances were perfused in the external solution.

Whole-cell patch-clamp recordings of I_h from nodose and trigeminal ganglion neurons in culture were made. Neurons were held at -40 mV (positive to I_h activation) and stepped to hyperpolarizing potentials until steady state currents were attained. Drugs were perfused or added to the internal solution. Two experimental protocols were used: a current/voltage protocol and a two-step time protocol. Activation plots were made from tail current amplitudes measured at -60 mV evoked by prepulses to a number of hyperpolarized potentials using the current/voltage protocol. The tail currents were normalized to the maximum control amplitude (I/I max(control)). The second experimental protocol, the two-step time protocol, used a prepulse from Vhold = -40 mV to -70 mV to activate I_h and a step back to -60 mV to elicit the tail current repeated every 30 s to determine the time course of I_h modulation by various drugs. Holding currents at -40 mV and tail currents evoked at -60 mV were plotted versus time. Results from drug applications were

expressed as the percent change from control.

Data analysis. Data were collected via an Axopatch 1-D amplifier and filtered at 2 kHz with a Bessel filter. Currents were digitized and recorded with Pclamp software and analyzed using Axograph (Axon Instruments, Inc.) and Kaleidograph software. Activation curves were fitted with a Boltzmann function using a least-squares algorithm to estimate the half-maximal voltage of activation (V1/2), maximum amplitude and slope values (Kaleidograph, Synergy Software). Descriptive statistics used were the mean and standard error of the mean. Paired t-tests were used to determine statistical significance of the effects of forskolin and PGE2 on control parameters. One-way ANOVAs were used to compare results between cells with control, C subunit, PKI, phosphatase inhibitors, RP-cAMP-S, or SP-cAMP-S in the internal pipette solution. Dunnett's post-hoc comparison test was used to determine the statistical significance of comparisons between control and other internal pipette solutions. A repeated ANOVA was used to determine if Boltzmann curves from cells recorded with control, RP-cAMP-S or SP-cAMP-S were significantly different. A two-way ANOVA was used to analyze the statistical difference of results from cells recorded with different Ca^{2+} internal solutions and treated with control, forskolin or PGE2 external solutions. The Scheffe post-hoc test was used to compare all possible combinations of means from the repeated and two-way ANOVAs. $p < 0.05$ was taken to indicate statistical significance in all tests.

RESULTS

Recordings were made from approximately 212 neurons. Once the whole-cell recording was established, a slow decline in the outward holding current at Vhold = -40 mV was apparent during the first five minutes. This was probably due to the block of K^+ currents resulting from the diffusion of Cs^+ into the cell. I_h generally activated between -50 mV and -70 mV and reached maximal amplitude at -100 to -110 mV (tail currents at -60 mV overlapped at these potentials). External Cs^+ (2 mM), an I_h blocker, blocked all of the

time-dependent inward current elicited with steps to hyperpolarized potentials.

Rundown of I_h was noted in most cells and was associated with both a decrease in maximum amplitude and a shift of the activation curve to more hyperpolarized potentials. The time course and extent of the rundown was extremely variable between cells. In an attempt to understand the underlying mechanism of run-down, the changes in V1/2, slope and maximal amplitude values as estimated from Boltzmann fits were calculated over a ten minute period (15 minute time point minus the 5 minute time point). The V1/2 shifted in the hyperpolarizing direction by 5 ± 1 mV and the maximum amplitude decreased by $10 \pm 3\%$ in cells recorded with control internal solution ($n = 5$). The negative shift of V1/2 was not significantly different in cells with any of the cAMP pathway modulators applied to the internal solution (Dunnett's, $p > 0.05$). However, the negative shift in maximum amplitude associated with run-down was significantly greater in cells recorded with okadaic acid (1 μM) in the internal pipette solution ($39 \pm 11\%$; $n = 3$) compared to controls (Dunnett's, $p < 0.05$). Thus, the V1/2 and maximum amplitude variables may be regulated by different mechanisms. Forskolin and PGE2 were applied 15 minutes after the onset of recording so that internal Cs^+ would be equilibrated and the amount of rundown could be assessed. After wash-out of forskolin and PGE2, I_h often over-recovered so that activation curves of each cell were more hyperpolarized than in control. However, in spite of rundown, responses to both PGE2 and forskolin could be elicited repeatedly (see Figure 1).

Prostaglandin E2 and forskolin shifted the voltage-dependence and increased the amplitude of I_h.

PGE2 (1 μM) shifted the voltage dependence of activation of I_h to more depolarized potentials (4 ± 1 mV; $n = 5$) and increased the maximum amplitude of I_h ($18 \pm 5\%$; $n = 5$) (Figure 1*A*) in 5/13 cells tested with control internal solution. Thus, only a sub-population of these neurons respond to PGE2 suggesting that not all cells express prostaglandin receptors. Forskolin (10 μM) shifted the voltage dependence of I_h in all cells tested under control conditions (5 ± 1 mV; $n = 6$) and increased the amplitude of I_h ($21 \pm 5\%$; $n = 6$).

There were no differences in slope values during application of forskolin or PGE2. Figure 1*B* shows the effects of both repeated forskolin applications and a PGE2 application on the I_h current evoked with the 2-step protocol (a step from -40 mV to -70 mV and back to -60 mV every 30 s). Although the amplitude of I_h runs down over the course of the experiment, forskolin and PGE2 responses could be repeated. External Cs^+ completely blocked all of the inward current, indicating the isolation of I_h in the presence of internal CsGluconate.

Role of phosphorylation by protein kinase A (PKA).

Forskolin and PGE2 increase the levels of cAMP in primary afferent neurons suggesting that the modulation of I_h may involve the cAMP second messenger pathway. In order to test the role of PKA, the cAMP analogs RP-cAMP-S and SP-cAMP-S were used in the internal pipette solution. RP-cAMP-S inhibits PKA, while SP-cAMP-S activates the enzyme. In Figure 2*A*, cells with internal RP-cAMP-S (1 mM) have activation curves that are shifted to significantly depolarized potentials (-60 ± 0.4 mV; n = 4) over control (-71 ± 1 mV; n = 4) 5 minutes after the whole-cell recording was established. The continuous 2-step protocol with prepulses to -70 mV showed that the augmentation of I_h by PGE2 and forskolin was occluded in these cells (n = 5; Figure 2*B*). Application of Cs^+ (2 mM) to the external solution completely blocked the inward current, suggesting that RP-cAMP-S had augmented I_h.

As with RP-cAMP-S, the cells with internal SP-cAMP-S (1 mM) had significantly depolarized activation curves compared to cells with control internal solution (-59 ± 0.3 mV; n = 4; Figure 3*A*). Similarly, forskolin no longer augmented I_h in cells with internal SP-cAMP-S (Figure 3*B*), and external Cs^+ blocked the entire inward current elicited by the hyperpolarizing step. Since forskolin always augmented I_h in control cells, even in the presence of marked rundown, the absence of forskolin responses during perfusion with RP-cAMP-S and SP-cAMP-S was due to occlusion.

The concentration dependence of RP-cAMP-S and SP-cAMP-S modulation was studied to determine if lower concentrations could differentiate the effects of these analogs on I_h. Only the highest concentration of RP-cAMP-S (1 mM; n = 4) and SP-cAMP-S (1 mM; n = 5) shifted the activation curves of I_h to significantly more depolarized potentials. However, concentrations of analogs above 10 μM significantly occluded the forskolin-induced shift of I_h activation (Figure 4). Lower concentrations had no effect. Although RP-cAMP-S inhibits and SP-cAMP-S activates PKA, they had similar effects on I_h at all concentrations, suggesting that modulation of I_h occurs through a direct action of cyclic nucleotides. Therefore, PKA is probably not involved in the tonic or forskolin-mediated modulation of I_h.

Cyclic nucleotides modulate I_h in primary afferents.

Cyclic nucleotide-gated channels in other sensory neurons can be selective for cAMP, cGMP or non-selective. The ability of cAMP and cGMP analogs to affect I_h was investigated to determine the cyclic nucleotide selectivity for I_h. Figure 5 shows that 8Br-cAMP and 8Br-cGMP (100 μM and 1 mM) shifted the voltage dependence of I_h to more depolarized potentials but had little effect at 10 μM. There was also an increase in maximum amplitude of the I_h activation curve in the presence of 8Br-cAMP (1 mM; 12 ± 6%; n =5) and 8Br-cGMP (1 mM; 12 ± 4%; n = 11). The slope values did not change consistently in the presence of the analogs. The effects of 8Br-cAMP and 8Br-cGMP were long-lasting. It often took twice as long to wash out 8Br-cAMP and 8Br-cGMP (1 mM) than to wash out forskolin and there was rarely an effect of a second application of cAMP or cGMP analogs, suggesting that the effects of these analogs are very slow to reverse. In fact, after a 10 minute wash-out period of 1 mM 8Br-cAMP or 8Br-cGMP, forskolin had no effect. Forskolin was effective, however, 10 minutes after washing lower concentrations of 8Br-cAMP and 8Br-cGMP (10 and 100 μM). The results of perfusion of other substances are included in Figure 5C. CPT-cAMP (1 mM; n = 4) shifted the voltage dependence of activation to more depolarized potentials, but db-cGMP (1 mM; n = 5), 5' AMP (1 mM; n = 5) and adenosine (1 mM; n = 4) did not.

Effects of other PKA modulators

To further test the possibility that PKA is involved in the tonic or receptor-mediated modulation of I_h, PKI (20 μM), C subunit (1.5 μM), microcystin (2 μM) and okadaic acid (1 μM) were applied via the internal solution and compared to cells with internal RP-cAMP-S and SP-cAMP-S. Only RP-cAMP-S (n = 5) and SP-cAMP-S (n = 6) significantly shifted the I_h activation curve to more depolarized potentials than control cells (n = 5) after the 5 minute equilibration period (Dunnett's, $p < 0.05$). After 15 minutes, activation curves for cells with C subunit (n = 5) and okadaic acid-containing (n = 3) internal solutions were significantly hyperpolarized compared to control, but SP-cAMP-S was still depolarized (Dunnett's, $p < 0.05$). These results suggest that PKA phosphorylation may be involved in the rundown process, but the mechanisms are not clear.

The effects of forskolin in the presence of these modulators was assessed by applying forskolin 15 minutes after the initiation of the recording in the same cells described above. Forskolin shifted the voltage dependence of I_h to more depolarized potentials in control, PKI, C subunit and okadaic acid-containing internal solutions but was not effective in cells with RP-cAMP-S and SP-cAMP-S in the internal solution. The results suggest that I_h was maximally shifted in the presence of the cAMP analogs (Figure 6).

The 2-step time protocol was used to determine if PKI, C subunit, okadaic acid or microcystin could modulate forskolin-induced I_h tail current amplitudes elicited by steps near the half-activation voltage (-70 mV) over time (Figure 6). Again, the results were consistent with results from current-voltage plots; RP-cAMP-S and SP-cAMP-S occlude the action of forskolin on I_h. PKI, C subunit and the phosphatase inhibitors did not significantly alter the response to forskolin.

Role of Ca^{2+} in PGE2 response

In the heart, I_h has been shown to be stimulated by increased intracellular calcium.

Experiments were performed with BAPTA (20 mM) to buffer the internal solution to pCa 10 and pCa 7 in order to test the possibility that I_h in primary afferents is also sensitive to intracellular calcium. There was no significant difference in the V1/2 values between pCa 7 (-79 ± 2 mV; n = 9) and pCa 10 (-79 ± 2 mV; n = 9) after 5 minutes of recording, suggesting that there is no tonic regulation of I_h by calcium in primary afferents. Forskolin shifted the voltage-dependence of I_h to the right by 3 ± 1 mV and increased the maximum amplitude by 6 ± 4% in high Ca^{2+} (pCa 7) internal solution (n =5) and 6 ± 2 mV and 15 ± 8% in low Ca^{2+} (pCa 10) internal solution (n = 6). PGE2 shifted the voltage-dependence of I_h to the right by 5 ± 2 mV and increased the maximum amplitude by 30 ± 17% in high Ca^{2+} (pCa 7) internal solution (n = 4) and 6 ± 2 mV and 2 ± 6% in low Ca^{2+} (pCa 10) internal solution (n = 4). None of these changes were significantly different between high Ca^{2+}, low Ca^{2+} or control internal solutions (Scheffe test, $p > 0.05$). There was also no difference in activation parameters during rundown (15 minutes minus 5 minutes) between different Ca^{2+} conditions. Therefore, calcium does not seem to be involved in either tonic regulation of I_h or in the ability of forskolin or PGE2 to stimulate I_h.

DISCUSSION

Cell type

Forskolin mimics the PGE2-induced shift in I_h activation in trigeminal and nodose ganglion primary afferents. PGE2 modulation occurred in a subpopulation of medium to large-sized neurons in the nodose ganglion and medium-sized cells in the trigeminal ganglion. Therefore, primary afferent neurons are probably heterogeneous with respect to expression of prostaglandin receptors coupled to activation of adenylyl cyclase. Primary afferent neurons have already been shown to be heterogeneous with respect to expression of I_h. Small-diameter neurons (C-fibers) in the dorsal root ganglion do not express I_h (Tokimasa, Shiraishi & Akasu, 1990), an observation confirmed in the nodose and trigeminal ganglia in these studies. In addition, neurons that expressed I_h had short duration action potentials that are indicative of Aδ-type cells (Scroggs, Todorovic, Anderson & Fox, 1994). The

nodose ganglion consists primarily of neurons with C- and Aδ-fibers, so I_h modulation probably occurs in Aδ-fiber neurons.

I_h type

PGE2 and forskolin increase cAMP in primary afferent neurons. Other neurotransmitter receptors coupled to G proteins which stimulate adenylyl cyclase shift the activation curve of I_h to more depolarized potentials in the heart (DiFrancesco, et al., 1986), bull-frog sympathetic neurons (Tokimasa & Akasu, 1990) and central neurons (Bobker & Williams, 1989; McCormick & Pape, 1990b; Banks, et al., 1993). Neurotransmitter receptors coupled to inhibitory G proteins that decrease cAMP levels in cells, such as muscarinic M2 receptors in the heart (DiFrancesco & Tromba, 1988; DiFrancesco, et al., 1989; Chang & Cohen, 1992) and μ-opioids in primary afferents (Ingram & Williams, 1994), shift the voltage dependence of I_h in the hyperpolarizing direction. Although I_h has been described in many cell types, there are some qualitative differences that suggest that I_h may be modulated differently in different tissues. I_h is a nonselective cation current that activates with hyperpolarizing voltage steps and is blocked by external Cs^+, but the activation range, amplitude, and modulation of I_h vary in different preparations. ß-adrenergic agonists and forskolin (DiFrancesco et al., 1986) shift the activation curve of I_h without an increase in maximum amplitude in SA node myocytes, but PGE2 and forskolin actually increase both of these parameters in primary afferent neurons. These results are consistent with the observations of Tokimasa & Akasu (1990) in sympathetic neurons. In addition, PKA phosphorylation (Chang, et al., 1991) has been proposed as the mechanism of I_h regulation in heart Purkinje fibers, but results refuting the role of PKA in SA node myocytes (Yatani, et al., 1990; DiFrancesco & Tortora, 1991) suggest that second messenger modulation of I_h is not the same in all cells. In light of these conflicting observations, it is important to understand the mechanism by which cAMP modulates I_h in primary afferents.

Phosphorylation or direct action of cAMP?

Although earlier studies with protein kinase inhibitors (H-7, H-8) suggested that PKA

phosphorylation was involved in the tonic modulation of I_h (Tokimasa & Akasu, 1990; Chang, et al., 1991), the inhibitors were very non-selective. RP-cAMP-S and SP-cAMP-S were used in the present studies to determine if PKA was involved in the augmentation of I_h in primary afferents because they are cAMP analogs that selectively inhibit and activate PKA, respectively. The surprising result was that both analogs augmented I_h and occluded stimulation of I_h by forskolin and PGE2. There were no differences at any concentration of RP-cAMP-S or SP-cAMP-S that could be attributed to either inhibition or activation of PKA, supporting the hypothesis that I_h in primary afferents is directly regulated by cAMP. This observation was confirmed by studies using PKI, C subunit, and phosphatase inhibitors (okadaic acid and microcystin) in that none of these substances shifted the activation curve of I_h to depolarized potentials or blocked the effects of forskolin. The results of the present study are consistent with results from single-channel and inside-out patch recordings from SA node cells myocytes showing direct modulation of I_h by cAMP (DiFrancesco & Tortora, 1991; DiFrancesco & Mangoni, 1994). Patches of primary afferents neurons with I_h have altered kinetics immediately after pulling the patch and run-down very quickly (unpublished observations). Therefore, RP-cAMP-S and SP-cAMP-S were the best tools to use in the present experiments. Although negative results with PKI and C subunit applied via the internal solution were a concern because there were no obvious positive controls, there are several reasons to believe that diffusion of these substances into the cell occurred. Electrode resistances were small (2-3 MΩ) and a five minute equilibration period was more than sufficient to observe the effects of internal RP-cAMP-S and SP-cAMP-S on I_h. There was also a significant effect of C subunit on the rate of rundown, suggesting the possibility that PKA is involved in run-down or some other aspect of tonic maintenance of I_h.

Cyclic-nucleotide-gated channels

In these studies, I_h was modulated by both cAMP and cGMP analogs. 8Br-cGMP (1 mM) shifted the voltage-dependence and increased maximum amplitude of I_h to the same extent as the cAMP analogs. db-cGMP was ineffective in these experiments, but db-cAMP was much less effective than CPT-cAMP or 8Br-cAMP in augmenting I_h in a previous study

(Ingram & Williams, 1994). Therefore, there may be some selectivity between analogs. Cyclic nucleotide-gated channels have been described from vertebrate retinal and olfactory sensory neurons (for review, Kaupp, 1991). The major difference between these channels is the selectivity for cyclic nucleotides. The retinal cyclic nucleotide-gated channel is approximately 30-fold more selective for cGMP (Kaupp, et al., 1989; Goulding, et al., 1992), and the olfactory cyclic nucleotide-gated channels are opened in the presence of micromolar concentrations of both cAMP and cGMP (Nakamura & Gold, 1987). The results of this present study suggest that I_h is regulated by 8Br-cAMP and 8Br-cGMP at similar concentrations and may be related to the olfactory cyclic nucleotide-gated channel. However, the cyclic nucleotide-gated channels are actually gated by cyclic nucleotides, while I_h seems to be gated by voltage and modulated by cyclic nucleotides. I_h has also been described in rod photoreceptors but modulation by cyclic nucleotides has not been addressed (Bader, Bertrand & Schwartz, 1982). Since photoreceptor responses to light are regulated by cGMP, it would be interesting to determine if I_h in these cells is also regulated by cGMP.

I_h in SA node cells has been shown to be sensitive to changes in internal Ca^{2+} (Hagiwara & Irisawa, 1989). There are prostaglandin receptor subtypes localized to dorsal root and trigeminal ganglion neurons that activate phospholipase C or adenylyl cyclase (Sugimoto, et al., 1994). Thus, PGE2 may be able to act on primary afferents through stimulation of internal Ca^{2+}. Our results indicate that internal Ca^{2+} concentrations buffered to the same concentrations used by Hagiwara & Irisawa (1989) had no effect on I_h activation parameters or run-down. There were also no significant effects of internal calcium on forskolin and PGE2 responses, although there was a hint of a difference between high and low calcium concentrations in the ability of PGE2 to increase the maximum amplitude. Thus, the main effects of PGE2 in primary afferent neurons are probably via activation of a prostaglandin receptor subtype coupled to stimulation of adenylyl cyclase. This is consistent with recent studies that show that one of the prostaglandin receptor subtypes, EP3, mediates peripheral hyperalgesia (Minami, Nishihara, Uda, Hyodo & Hayaishi,

1994) through an increase in cAMP (Khasar, Ouseph, Chou, Ho, Green & Levine, 1995) .

Significance

PGE2 increases excitation and sensitizes small-diameter primary afferents (Handwerker, 1976; Schaible & Schmidt, 1988). Increases in cAMP are associated with pain and hyperalgesia (Taiwo, Bjerknes, Goetzl & Levine, 1989), and activation of adenylyl cyclase is thought to be the mechanism by which prostaglandins released during the inflammatory response produce hyperalgesia (Ferreira & Nakamura, 1979). Modulation of I_h by cAMP leads to increased spontaneous firing of SA node cells of the heart (Brown & DiFrancesco, 1980; DiFrancesco, 1991; Noble, et al., 1992) and central neurons (McCormick & Pape, 1990b). Therefore, because forskolin and PGE2 shift the voltage dependence and increase I_h amplitude through stimulation of adenylyl cyclase, augmentation of I_h may lead directly to excitation of primary afferents and increased pain. Alternatively, as PGE2 is a potent hyperalgesic agent, augmentation of I_h may underly the PGE2 -induced depolarization observed in primary afferents by Yanagisawa, Otsuka & García-Arrarás (1986) that results in sensitization to subsequent stimuli.

REFERENCES

Bader, C. R., Bertrand, D. & Schwartz, E. A. (1982). Voltage-activated and calcium-activated currents studied in solitary rod inner segments from the salamander retina. *Journal of Physiology* **331**, 253-284.

Banks, M. I., Pearce, R. A. & Smith, P. H. (1993). Hyperpolarization-activated cation current (I_h) in neurons of the medial nucleus of the trapezoid body: voltage-clamp analysis and enhancement by norepinephrine and cAMP suggest a modulatory mechanism in the auditory brain stem. *Journal of Neurophysiology* **70**, 1420-1432.

Beech, D. J., Bernheim, L., Mathie, A. & Hille, B. (1991). Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons. *Proceedings of the National Academy of Sciences USA* **88**, 652-656.

Benham, C. D., Bolton, T. B., Denbigh, J. S. & Lang, R. J. (1987). Inward rectification in freshly isolated single smooth muscle cells of the rabbit jejunum. *Journal of Physiology* **383**, 461-476.

Bobker, D. H. & Williams, J. T. (1989). Serotonin augments the cationic current I_h in central neurons. *Neuron* **2**, 1535-1540.

Brown, H. & DiFrancesco, D. (1980). Voltage-clamp investigations of membrane current underlying pace-maker activity in rabbit sino-atrial node. *Journal of Physiology* **308**, 331-351.

Chang, F. & Cohen, I. S. (1992). Mechanism of acetylcholine action on pacemaker current (i_f) in canine Purkinje fibers. *Pflügers Archives* **420**, 389-392.

Chang, F., Cohen, I. S., DiFrancesco, D., Rosen, M. R. & Tromba, C. (1991). Effects of

protein kinase inhibitors on canine Purkinje fibre pacemaker depolarization and the pacemaker current i_f. *Journal of Physiology* **440**, 367-384.

Cui, M. & Nicol, G, D. (1995). Cyclic AMP mediates the prostaglandin E_2-induced potentiation of bradykinin excitation in rat sensory neurons. *Neuroscience* **66**, 459-466.

Denyer, J. C. & Brown, H. F. (1990). Pacemaking in rabbit isolated sino-atrial node cells during Cs^+ block of the hyperpolarization-activated current i_f. *Journal of Physiology* **429**, 401-409.

DiFrancesco, D. (1991). The contribution of the 'pacemaker' current (i_f) to generation of spontaneous activity in rabbit sino-atrial node myocytes. *Journal of Physiology* **434**, 23-40.

DiFrancesco, D., Ducouret, P. & Robinson, R. B. (1989). Muscarinic modulation of cardiac rate at low acetylcholine concentrations. *Science* **243**, 669-671.

DiFrancesco, D., Ferroni, A., Mazzanti, M. & Tromba, C. (1986). Properties of the hyperpolarizing-activated current (i_f) in cells isolated from the rabbit sino-atrial node. *Journal of Physiology* **377**, 61-88.

DiFrancesco, D. & Mangoni, M. (1994). Modulation of single hyperpolarization-activated channels (*if*) by cAMP in the rabbit sino-atrial node. *Journal of Physiology* **474**, 473-482.

DiFrancesco, D. & Tortora, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature* **351**, 145-147.

DiFrancesco, D. & Tromba, C. (1988). Muscarinic control of the hyperpolarization-activated current (i_f) in rabbit sino-atrial node myocytes. *Journal of Physiology* **405**, 493-

510.

Ferreira, S. H. & Nakamura, M. (1979). I - Prostaglandin hyperalgesia, a cAMP/Ca^{2+} dependent process. *Prostaglandins* **18**, 179-190.

Goulding, E. H., Ngai, J., Kramer, R. H., Colicos, S., Axel, R., Siegelbaum, S. A. & Chess, A. (1992). Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons. *Neuron* **8**, 45-58.

Hagiwara, N. & Irisawa, H. (1989). Modulation by intracellular Ca^{+2} of the hyperpolarization-activated inward current in rabbit single sino-atrial node cells. *Journal of Physiology* **409**, 121-141.

Handwerker, H. O. (1976). Influences of algogenic substances and prostaglandins on the discharges of unmyelinated cutaneous nerve fibers identified as nociceptors. *Advances in Pain Research and Therapy* **1**, 41-45.

Hingtgen, C. M., Waite, K. J. & Vasko, M. R. (1995). Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3', 5'-cyclic monophosphate transduction cascade. *Journal of Neuroscience* **15**, 5411-5419.

Ingram, S. L. & Williams, J. T. (1994). Opioid inhibition of I_h via adenylyl cyclase. *Neuron* **13**, 179-186.

Kamondi, A. & Reiner, P. B. (1991). Hyperpolarization-activated inward current in histaminergic tuberomammillary neurons of the rat hypothalamus. *Journal of Neurophysiology* **66**, 1902-1911.

Kaupp, U. B. (1991). The cyclic nucleotide-gated channels of vertebrate photoreceptors

and olfactory epithelium. *Trends in Neurosciences* **14**, 150-157.

Kaupp, U. B., Niidome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N. J., Kangawa, K., Matsuo, H., Hirose, T., Miyata, T. & Numa, S. (1989). Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic-GMP-gated channel. *Nature* **342**, 762-766.

Khasar, S. G., Ouseph, A. K., Chou, B., Ho, T., Green, P. G. & Levine, J. D. (1995). Is there more than one prostaglandin E receptor subtype mediating hyperalgesia in the rat hindpaw? *Neuroscience* **64**, 1161-1165.

Mayer, M. L. & Westbrook, G. L. (1983). A voltage-clamp analysis of inward (anomalous) rectification in mouse spinal sensory ganglion neurones. *Journal of Physiology* **340**, 19- 45.

McCormick, D. A. & Pape, H. -C. (1990a). Properties of a hyperpolarization-activated cation current and its role in rhythmic oscillation in thalamic relay neurones. *Journal of Physiology* **431**, 291-318.

McCormick, D. A. & Pape, H. -C. (1990b). Noradrenergic and serotonergic modulation of a hyperpolarization-activated cation current in thalamic relay neurones. *Journal of Physiology* **431**, 319-342.

Minami, T., Nishihara, I., Uda, R., Ito, S., Hyodo, M. & Hayaishi, O. (1994). Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglandin E2 to mice. *British Journal of Pharmacology* **112**, 735-740.

Nakamura, T. & Gold, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325**, 442-444.

Noble, D., Denyer, J. C., Brown, H. F. & DiFrancesco, D. (1992). Reciprocal role of the inward currents $i_{b,Na}$ and i_f in controlling and stabilizing pacemaker frequency of rabbit sino-atrial node cells. *Proceedings of the Royal Society of London, Biology* **250**, 199-207.

Schaible, H. -G. & Schmidt, R. F. (1988). Excitation and sensitization of fine articular afferents from cat's knee joint by prostaglandin E_2. *Journal of Physiology* **403**, 91-104.

Scroggs, R. S., Todorovic, S. M., Anderson, E. G. & Fox, A. P. (1994). Variation in I_H, I_{IR}, and I_{LEAK} between acutely isolated adult rat dorsal root ganglion neurons of different size. *Journal of Neurophysiology* **71**, 271-279.

Sugimoto, Y., Shigemoto, R., Namba, T., Negishi, M., Mizuno, N., Narumiya, S. & Ichikawa, A. (1994). Distribution of the messenger RNA for the prostaglandin E receptor subtype EP_3 in the mouse nervous system. *Neuroscience* **62**, 919-928.

Taiwo, Y. O., Bjerknes, L. K., Goetzl, E. J. & Levine, J. D. (1989). Mediation of primary afferent hyperalgesia by the cAMP second messenger system. *Neuroscience* **32**, 577-580.

Taiwo, Y. O. & Levine, J. D. (1991). Prostaglandin effects after elimination of indirect hyperalgesic mechanisms in the skin of the rat. *Brain Research* **492**, 397-399.

Tokimasa, T. & Akasu, T. (1990). Cyclic AMP regulates an inward rectifying sodium-potassium current in dissociated bull-frog sympathetic neurones. *Journal of Physiology* **420**, 409- 429.

Tokimasa, T., Shiraishi, M. & Akasu, T. (1990). Morphological and electrophysiological properties of C-cells in bullfrog dorsal root ganglia. *Neuroscience Letters* **116**, 304-308.

Yanagihara, K. & Irisawa, H. (1980). Inward current activated during hyperpolarization in

the rabbit sinoatrial node cell. *Pflügers Archives* **385**, 11-19.

Yanagisawa, M., Otsuka, M & García-Arrarás, J. E. (1986). E-type prostaglandins depolarize primary afferent neurons of the neonatal rat. *Neuroscience Letters* **68**, 351-355.

Yatani, A., Okabe, K., Codina, J., Birnbaumer, L. & Brown, A. M. (1990). Heart rate regulation by G proteins acting on the cardiac pacemaker channel. *Science* **249**, 1163-1166.

ACKNOWLEDGEMENTS

We thank Z. Hausken for helpful discussion, K. Drews for technical assistance, and N. Marrion and E. McCleskey for comments on this manuscript. This work was supported by NIH grants DA08163 and DA07262.

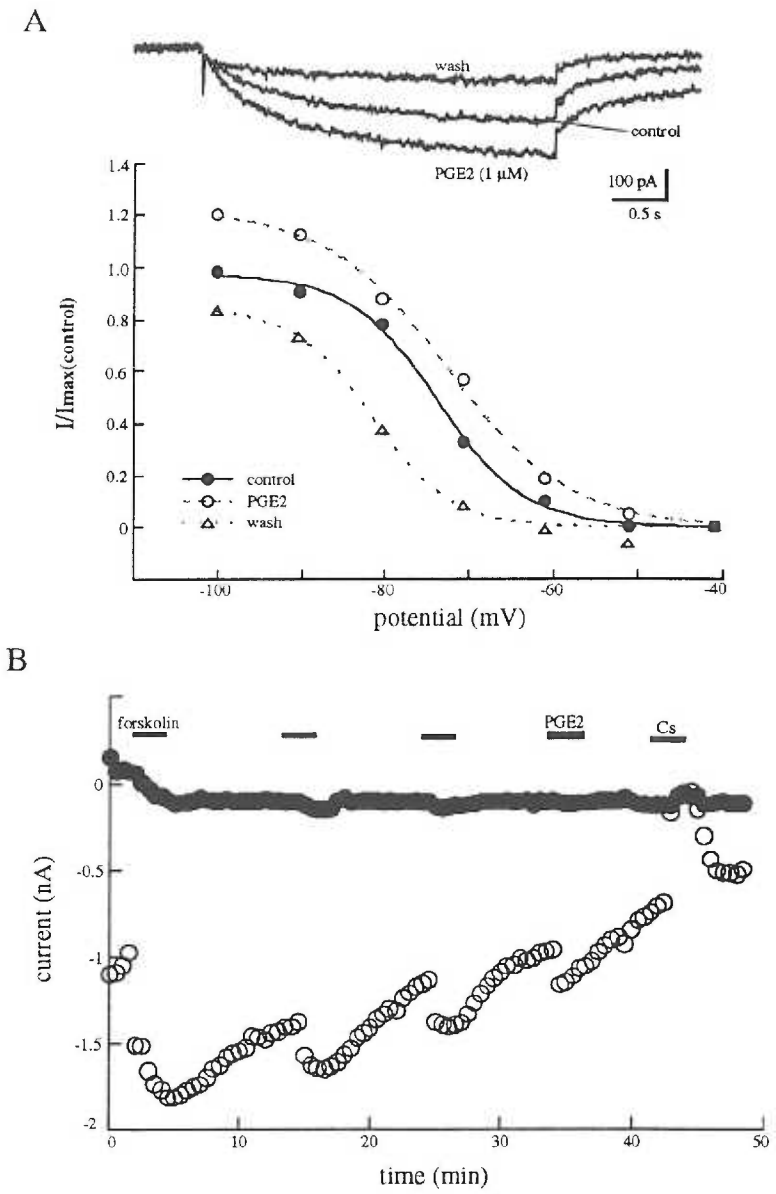

Figure 1. PGE2 and forskolin shift the voltage dependence and increase the amplitude of I_h. *A*, Superfusion of PGE2 (1 μM) increases the maximum amplitude and shifts the voltage dependence of I_h (V1/2) to more depolarized potentials. Representative traces of a step from -40 mV to -70 mV are depicted in inset. *B*, Change in I_h over time with 2-step protocol during applications of forskolin (10 μM) and PGE2 (1 μM). Steps were made from Vhold = -40 mV (filled circles) to -70 mV and tail currents were measured at -60 mV (open circles). The inward current was entirely I_h as external Cs^+ effectively blocked all of this current. Both forskolin and PGE2 augment I_h. The amount of run-down during the recording period is highly variable between cells.

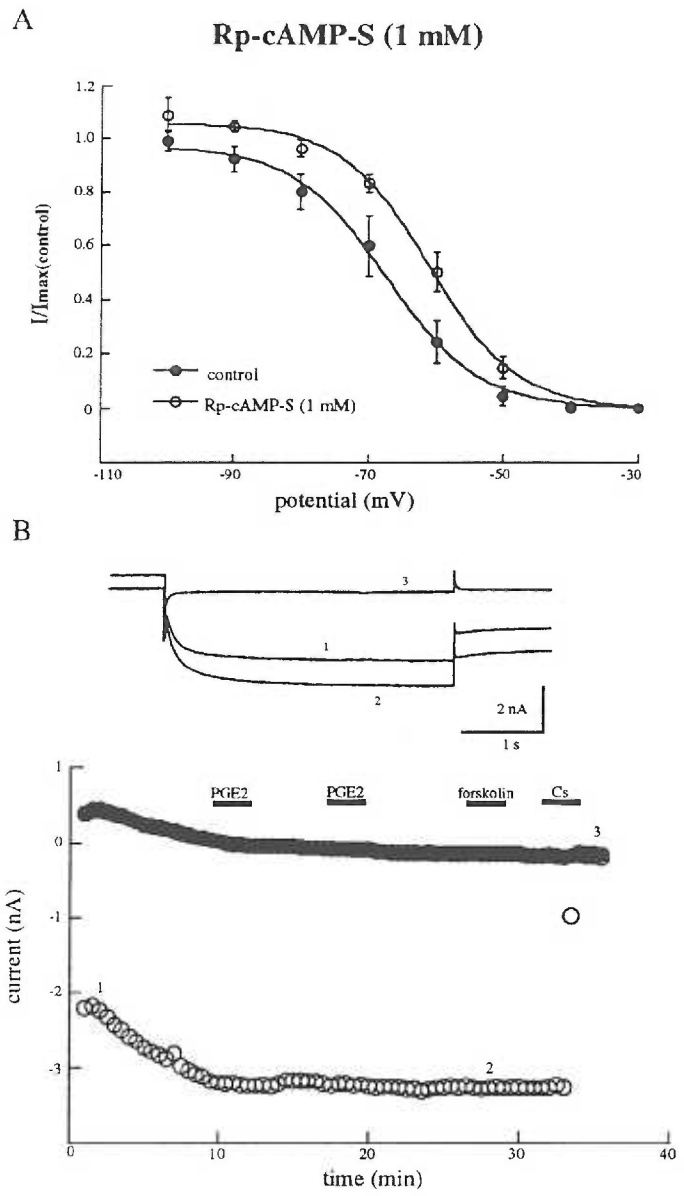

Figure 2. RP-cAMP-S modulates I_h and occludes forskolin and PGE2 effects. *A*, Averaged activation curves for I_h in the presence (n = 4) and absence (n = 4) of RP-cAMP-S (1 mM) are significantly different over the potential range. *B*, Steps from Vhold=-30 mV (filled circles) to -70 mV and back to -60 mV (open circles) were made every 30 s after break-in to the whole-cell mode. Representative traces are indicated by numbers to emphasize several points: the baseline shifts in the first five minutes, there is no effect of PGE2 (1 μM) or forskolin (10 μM), and as Cs^+ (2 mM) blocks the entire inward current, there is no indication that RP-cAMP-S activates another inward current.

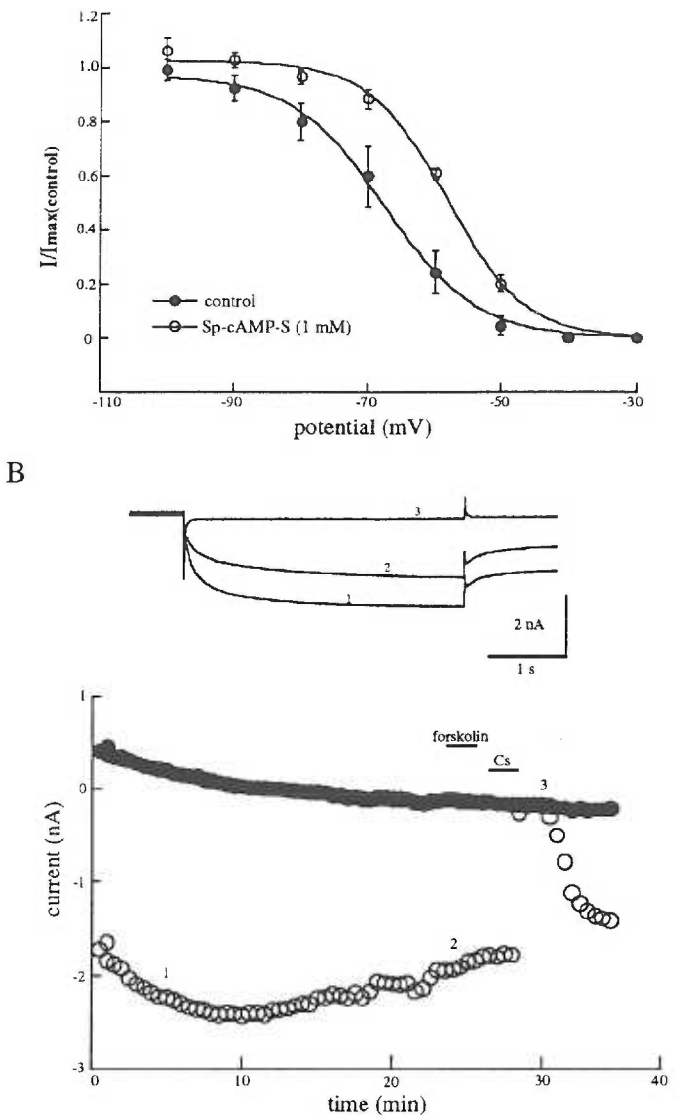

Figure 3. SP-cAMP-S modulates and occludes forskolin modulation of I_h. *A*, Averaged activation curves for I_h in the presence (n = 4) and absence (n = 4) of SP-cAMP-S (1 mM) showing the significant shift of activation five minutes after breaking into whole-cell mode. *B*, A recording with internal SP-cAMP-S (1 mM). Measurements were taken from tail currents at -50 mV (open circles) elicited by prepulses from Vhold = -30 mV (filled circles) to -60 mV. The forskolin (10 μM) response is occluded and Cs^+ blocks all of the inward current. Representative traces are taken from numbered time points. This cell showed run-down during the recording. Run-down was not seen in all cells with SP-cAMP-S and was also seen in some cells with RP-cAMP-S.

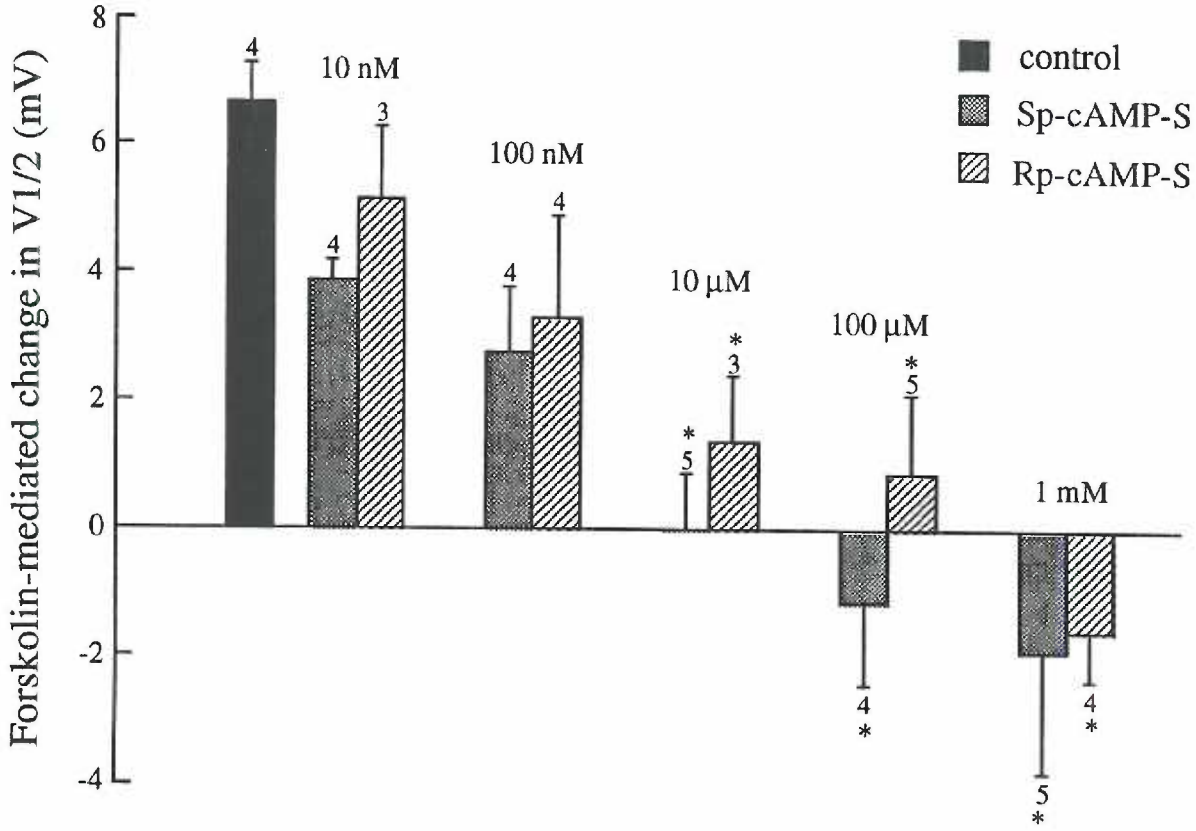

Figure 4. RP-cAMP-S and SP-cAMP-S have similar effects over a large range of concentrations. Forskolin shifts the voltage-dependence (V1/2) of I_h to depolarized potentials (positive direction) in control (filled bar). SP-cAMP-S (cross-hatched bars) and RP-cAMP-S (hatched bars) occlude the forskolin-mediated change in V1/2 compared to control in a concentration-dependent manner. *indicates a significant decrease in the forskolin effect from control.

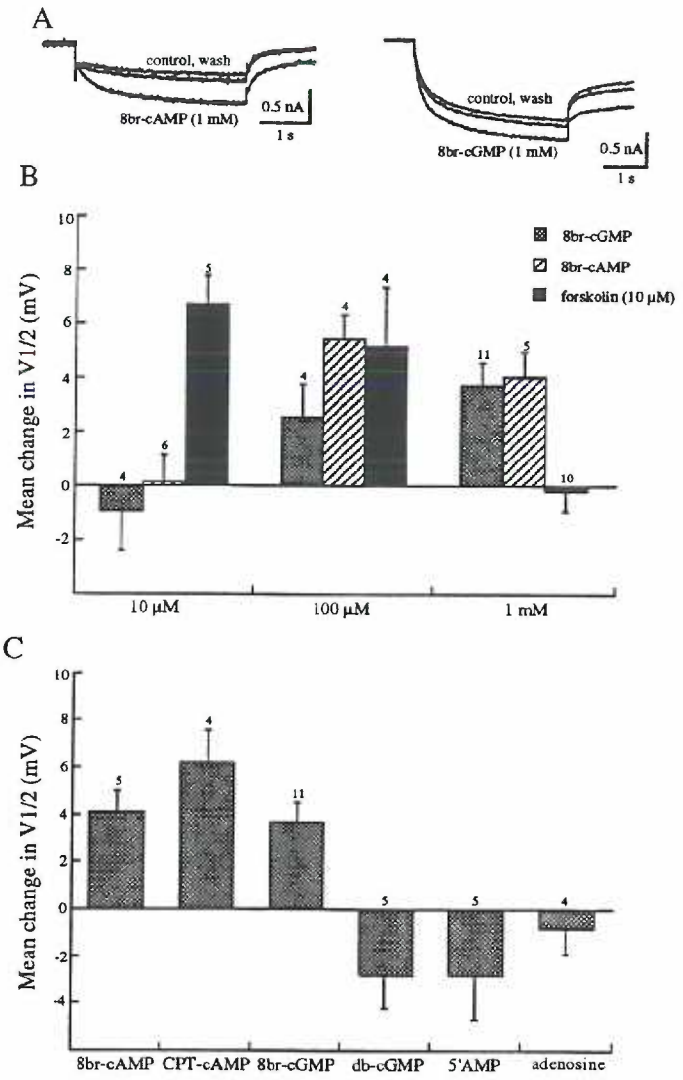

Figure 5. Cyclic nucleotides modulate I_h in primary afferents. *A*, Representative traces elicited by prepulses from -40 mV to -70 mV and tail currents at -60 mV from two different cells given 8Br-cAMP (1 mM) or 8Br-cGMP (1 mM). *B*, Bar graph showing the mean change in V1/2 during perfusion of different concentrations of 8Br-cAMP (hatched bars) and 8Br-cGMP (cross-hatched bars). Filled bars represent the shift in the activation curve by forskolin (10 µM) applied after a 10-minute wash-out period of the respective concentrations of analogs. *C*, Bar graph showing the mean change in V1/2 during perfusion of several cAMP and cGMP analogs as well as 5'AMP and adenosine. Data for the 8Br-cAMP and 8Br-cGMP (1 mM) are from same cells as above. Number of cells tested with each substance are indicated.

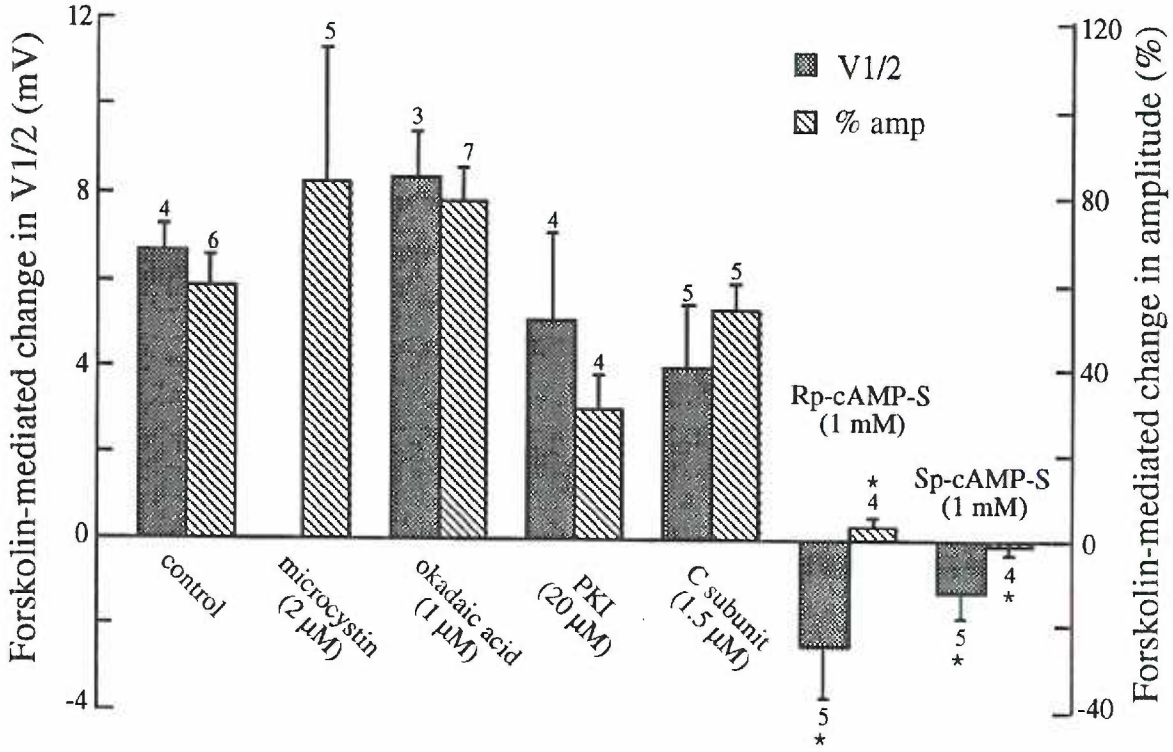

Figure 6. Effects of phosphorylation modulators on the forskolin-mediated change in V1/2 and % change in I_h amplitude. Inhibitors of PKA (PKI and RP-cAMP-S), activators of PKA (C subunit and SP-cAMP-S), and phosphatase inhibitors (microcystin and okadaic acid) were applied via the internal solution. Current/voltage protocols were run every 5 minutes and forskolin (10 μM) was applied after 15 minutes of recording. Forskolin was not effective in shifting the voltage dependence of I_h or the tail current amplitudes in cells with RP-cAMP-S or SP-cAMP-S in the internal solution. PKI, C subunit and the phosphatase inhibitors had no significant effect on either variable. Microcystin was not used in current/voltage protocol experiments.

Journal of Physiology (1995), 482.1

NOTICE TO CONTRIBUTORS, 1995

All submissions and enquiries should be sent to:

The Publications Office
The Journal of Physiology
Printing House
Shaftesbury Road
Cambridge CB2 2BS
United Kingdom

Tel: (01223) 68713
Fax: (01223) 312849
International: +44 1223 68713
Email: physio@cup.cam.ac.uk
Web: http://physiology.cup.cam.ac.uk/

Offprints of this notice may be obtained from the Secretary of the Editorial Board at the above address.

Policy

The Journal of Physiology publishes papers which present the authors' own original experimental work and which illustrate new physiological principles or mechanisms. As well as human and mammalian physiology this includes work at the level of the cell membrane, single cells, tissues or organs. Work on invertebrate or lower vertebrate preparations is suitable if it contributes results and further understanding applicable in general to the functioning of organisms including mammals.

Work on systems physiology of lower vertebrates and invertebrates, specific to that type of organism, is not suitable. Since there is great pressure on space in the journal some papers may be rejected on the grounds that the contribution they make is insufficient to justify publication. Clarity of expression and conciseness will also be taken into consideration.

Rapid publication of Short Papers

Authors may request that their manuscript be considered as a Short Paper for rapid publication. **The acceptance criteria for Short Papers reflect the expectation that these papers should be of particularly high interest and importance.** Such papers will be given a rapid review.

Papers should be complete in themselves. Pairs or sequences of papers are not allowed. Papers should be presented in the usual format, having a Summary, Introduction, Methods, Results and Discussion. The length of a short paper should not exceed 3500 words including figure legends (about twelve double-spaced manuscript pages at 12 c.p.i.). In addition up to four figures or tables and twenty references will be allowed. The other conditions of publication are as below. Submissions should be accompanied by the usual copyright declaration and copies of any other relevant documents.

Authors must state at the bottom of the title page and in the covering letter that they wish the manuscript to be considered as a Short Paper for rapid publication.

For Short Papers only, a copy of the manuscript should be sent on disk. A telephone and fax number, and email address if available, should be given, with the corresponding author's address.

Overlapping material

Only in exceptional circumstances may results submitted for publication in the journal repeat findings already published (or intended to be published) by the authors elsewhere. Authors should instead refer to their previous findings in the same way as they would if the work had come from a different group. This policy applies to results in the wider sense and not simply to figures or parts of figures. The Editorial Board emphasizes that *a manuscript which is merely an expanded version of work published elsewhere is not acceptable.* An exception to this policy applies in the case of an abstract which does not exceed about 400 words.

The submission must be accompanied by a copy of any of the authors' material that overlaps the content of the manuscript. This should include preliminary notes, communications, abstracts, chapters or reviews, published in the last year, in press or submitted by any of the authors. *It must also be accompanied by a declaration in the following terms*:

'The attached paper entitled ... does not contain material, other than that submitted with the manuscript, which has been, or is intended to be, published anywhere except in *The Journal of Physiology*. It is understood that upon acceptance for publication the entire copyright in this paper shall pass to The Physiological Society. I/We confirm that any material of mine/ours, published in the last year, in press or submitted, which overlaps in content with this manuscript or which is cited in this manuscript, accompanies this submission and is listed below. I/We also confirm that any acknowledgements of scientific assistance or advice and any references to unpublished material or personal communications have been seen and approved by those concerned.

List of overlapping material.'

The declaration must be signed by each author.

Administrative procedure

Following submission of a manuscript, all correspondence, up to the stage of acceptance, is dealt with by the Distributing Editor whose name, address, telephone and fax numbers are given in the letter of acknowledgement. After acceptance all matters related to printing are dealt with by the Secretary of the Editorial Board (address above).

The Editors cannot accept responsibility for damage to or loss of papers submitted to them. Contributors are advised to keep copies of the typescript and illustrations.

The date of receipt of each paper will be printed and is normally the date on which the manuscript is first received by the Editors. Papers submitted without all the information in the form requested in this notice will not be given editorial consideration until it has been provided; the date of receipt published will then refer to the date when a complete submission is received. For papers delayed in revision by the author for an excessive period (usually 6 months) the date published will refer to receipt of the revised version.

Proofs should be corrected and returned promptly since publication is in order of receipt of corrected proofs. Proofs are mainly for the purpose of correcting printing errors; excessive alterations due to amendment of the author's accepted copy may be charged to the author.

Fifty offprints are provided to authors free of charge. Further offprints can be supplied if ordered and paid for at the same time as proofs are returned.

Preparation of manuscripts

Papers sent for publication must be typewritten on one side of the page only, on sheets of good-quality paper of uniform size. This applies to all sections of the paper including Methods, References, figure legends, table headings and footnotes. Dot-matrix printer output must be of at least near-letter quality. Papers should be carefully corrected and as nearly ready for press as possible before submission. Insertions, which should be typewritten or in ink, should be few and legible. Authors should consult recent volumes of the journal in order to ascertain the general style of presentation.

Three complete copies of the paper (photocopies of high quality are acceptable) are required. Pages must be numbered.

Disks should **not** be submitted at this stage, except in the case of Short Papers. Authors will be asked to send the text on disk once the paper has been accepted, although clear typewritten papers will be accepted *if the author did not use a computer*. Authors should note that although any word-processing and graphics packages are suitable, desktop publishing (DTP) packages are *not*, except where they are able to produce plain ASCII, RTF, TXT or WP files.

Arrangement of papers

The usual format is:

1. Title page	6. Discussion
2. Summary	7. References
3. Introduction	8. Acknowledgements
4. Methods	9. Tables
5. Results	10. Figures and legends

Papers deviating from the usual format can be considered for publication if there are obvious and compelling reasons for the variation. The desired position of figures and tables should be indicated in the text.

1. Title page

The title should be on a separate sheet with the authors' names, the name and address of the laboratory where the work was carried out, a running title, key words and the name, address, telephone and fax number along with email address, if available, for correspondence and proofs.

The title of the paper should be as informative as possible, but it must not exceed 120 letters and spaces. The animal species normally appears in the title. The Editors will not accept a series of papers with the same main title, e.g. 'Studies in ... Part I', 'Studies in ... Part II'.

The following abbreviations are allowed in the title:

ATP, ADP, AMP, cAMP, GTP, GDP, GMP, cGMP, GTPγS, GDPβS, DNA, RNA, mRNA, GABA, NMDA, 5-HT, PGE, ACh, InsP_3, IP_3, AMPA;
Chemical symbols, e.g. K^+, Ca^{2+};
Certain common peptides, e.g. 'the peptide VIP', 'the peptide ACTH';
Common cell lines, e.g. MDCK, 3T3, HeLa;
Fluorescent indicators, e.g. 'the indicator SBFI';
EPSC, IPSC, EPSP, IPSP, NMR, pH, P_{O_2}, P_{CO_2}.

The address from which the paper originates should be the minimal adequate postal address with post or zip code.

A running title not exceeding 45 letters and spaces must be given for page headings; the abbreviations above may be used.

For the Subject Index to the journal, authors should provide up to three key words to indicate under which headings their paper should be listed; these should be chosen from those in the most recent Cumulative Index (to volumes 432–473).

2. Summary

The Summary is arranged in short numbered paragraphs. It contains a succinct account of the problem, the methods, the results and the conclusions. References may not be cited.

Since the Summary may be used by abstracting journals, a limit of 200 words is recommended. It must not exceed 5 % of the text (excluding references and figure legends).

3. Introduction

The Introduction makes clear the object of the research. Reference to the authors' own previous work is desirable only if it has a direct bearing on the subject of the paper; an extensive historical review is not appropriate.

4. Methods

Methods are described once only and do not appear in the legends of figures and tables. Sufficient details are given to allow the work to be repeated by others: the maker's name and address, including country, are given for non-standard chemicals, apparatus and equipment. Materials known by a trade name, e.g. Perspex, have the initial letter as a capital and, if the material is not in common use, the maker's name is given. The Latin names of non-mammalian species are given.

Where experiments involve the use of humans or animals authors should state if local or national guidelines have been followed and/or ethics committee approval obtained. For further instructions on the details required, see under 'Limitations to acceptance' below.

Text in this section appears in small print.

5. Results

Quantitative observations are often better presented graphically than in tables. Where numerical results are given the number of significant figures should be related to the accuracy of the results. It is not usually necessary to present the individual results of a large number of repeated tests if the number of measurements is stated. Standard deviations and standard errors should be given, when pertinent, with no more than two significant figures. Theory and inference must be clearly distinguished from what was observed, and should not be elaborated in this section.

6. Discussion

A Discussion follows the statement of Results and is separate from it. The assumptions involved in making inferences from the experimental results should be stated. The Discussion should not merely recapitulate the Results. Authors should provide a succinct conclusion to their work.

In appropriate circumstances an Appendix or a Theory section may be accepted where, for example, it is necessary to derive mathematical results required in the paper.

7. References

The paper concludes with a list of the papers and books cited in the text. The number of references must be kept to a minimum. It is rarely necessary to cite more than forty works. Journal titles are given in full, with capitalization of both nouns and adjectives. The order of references is strictly alphabetical, regardless of chronology. The style of citations may be ascertained from papers in this volume; attention to punctuation is required.

In the text, references should be made by giving the author and the year of publication in parentheses, e.g. (Hill, 1938), except when the author's name is part of the sentence, e.g. 'Hill (1938) showed that ...'. Where several references are given together they are in chronological order, separated by semicolons.

When a paper written by two authors is quoted, both names are given; for three to six authors all the names are given the first time the reference is cited, but thereafter the first name only is given, followed by '*et al.*'. This form is used for all citations of a paper with more than six authors. Unpublished material may be referred to sparingly in the text, by giving the author's name and initials followed by 'unpublished observation' or 'personal communication'; it should not appear in the list of references. The wording of such references must have been seen and approved by the persons concerned.

8. Acknowledgements

Acknowledgements should be the minimum consistent with courtesy. The wording of acknowledgements of scientific assistance or advice must have been seen and approved by the persons concerned.

9. Tables

Tables should be used sparingly. They should be typed double-spaced on separate numbered sheets and referred to in the text by arabic numerals, e.g. Table 3. The expression 'the following table' must be avoided. Each table has its own self-explanatory title. The same information should not be presented in both tabular and graphical forms. The units in which the results are expressed are given in parentheses at the top of each column and not repeated on each line of the table. Long descriptive headings to columns should be avoided; 'ditto' signs are not used.

10. Figures and legends

Figures should be given a title and be accompanied by legends which make them comprehensible without reference to the text, although undue repetition should be avoided. A set of labelled copies of the figures must be provided with each of the three copies of the paper; these copies should have their legends attached.

For clarity, it is recommended that symbols on graphs etc. should be chosen from the following:

○ □ △ ● ■ ▲ ◇ ◆ ☆ ★ ⊗ ⊖ ⊠ ⊕ ▽ ▼,

and that bars in histograms etc. should be distinguished using the following patterns:

▨ ▨ ▩ ▩ ■ □.

Reversed hatching (▧ ▧) and vertical or horizontal hatching is best avoided, as are greys. Other symbols and patterns may be used if more are required.

One original copy of each figure, as large as possible up to a maximum of 297 × 210 mm (A4), is required. These originals must be of a high standard; poor-quality photocopies, recordings on chart paper, low-resolution computer plots, and white-on-black figures are not acceptable. Avoid grey shading if possible. All originals should, preferably, be unlettered; lettering in journal style will be applied by the Publications Office. Boxes surrounding figures will be removed. In the case of half-tones and anatomical drawings (e.g. tracings of sections) originals **must** be unlettered but scale, bars (defined in the legend), and arrows should be set.

Authors are encouraged to submit coloured illustrations whenever they enhance the scientific value of the paper. Authors will normally be expected to pay a small proportion of the actual costs. Enquiries should be addressed to the Publications Office.

Text style

Papers must be written in English (spelling as in *The Chambers Dictionary* or the *Shorter Oxford English Dictionary*). Up to three levels of subheading may be used in addition to the section headings (such as RESULTS). Small print may be used sparingly. Footnotes are not acceptable.

Chemical and biological nomenclature

In general, the conventions in chemical nomenclature adopted by the Biochemical Society should be followed. These are described in the *Biochemical Journal* (1995) 305, 1–15.

Names of species and genera should be in italics (or underlined). Names of muscles, bones, etc., should be in roman type (i.e. not in italics).

Abbreviations

Abbreviations should be avoided unless they are easily understood and help in reading the paper, and they should be kept to a minimum. When first introduced, in the text or in labelling figures, abbreviations should be defined. Abbreviations are normally printed in upper-case letters without stops (e.g. EPSP, EMG, AHP), with certain well-established exceptions, e.g. S.E.M., S.D., p.p.m.

Symbols, units and mathematical notation

Recommended mathematical symbols and ways of printing them are given in *Quantities, Units, and Symbols* (1975), Royal Society, London, where there is also a full discussion of the use of units.

It is expected that authors will use the SI system of units. Certain traditional units which are still in common usage are also acceptable. As a guide the following are listed:

pressure, mmHg (SI unit kPa)
radioactivity, Ci = Curie (SI unit Bq)
O_2 uptake and CO_2 elimination, $l\ min^{-1}$ (SI unit $mol\ s^{-1}$).

Indexed notation is used, e.g. $ml\ s^{-1}$, rather than ml/s.

Symbols must always be defined. Primary symbols representing physical quantities are usually single letters printed in italics. Suffixes to these should be inferiors, e.g. V_{max}, not Vmax. When there are two or more suffixes, these should normally be set as inferiors, separated by commas, e.g. $V_{O_2,max}$ (with certain well-established exceptions, e.g. a^1_{Na}, $I_{K(Ca)}$).

Authors should ensure that consistency is maintained throughout the manuscript, including illustrations and tables.

Limitations to acceptance

1. Human experiments

Papers describing experiments on human subjects or patients, involving procedures which are not therapeutic and which carry a risk of harm, must include evidence that the experiments were performed with the understanding and consent of each individual and that the study had been approved by the local ethics committee. In the case of experiments involving minors, any risks must have been considered absent or minimal, and evidence must be presented that the experiments were performed with the understanding and consent of the legal guardian. In this connection, the Editors wish to draw the attention of authors to the *Code of Ethics of the World Medical Association* (Declaration of Helsinki) and to the Medical Research Council's pamphlet *Responsibility in Investigations on Human Subjects*. Both documents were reprinted in the issue of the *British Medical Journal* of 18 July 1964.

2. Animal experiments

The Editors will not allow the publication of papers describing experimental procedures on living animals which may reasonably be presumed to have inflicted unnecessary pain or discomfort upon them.

To be acceptable for publication, experiments on living vertebrates or *Octopus vulgaris* should conform to the legal requirements in the UK. Whenever appropriate, a statement should be included indicating that experiments were performed in accordance with local/national guidelines. Authors must provide a full description of their anaesthetic and surgical procedures. They must also provide evidence that they took adequate steps to ensure that animals did not suffer unnecessarily at any stage of the experiment.

Papers describing experiments on isolated tissues must indicate whether the donor animal was killed or anaesthetized, and how this was accomplished.

3. Use of muscle relaxants

In experiments involving the use of muscle relaxants authors must describe the precautions taken to ensure the adequacy of anaesthesia. They must provide sufficient detail to enable the reader to determine that no unnecessary suffering occurred. The Physiological Society has issued the following advice:

'When muscle relaxants are used with anaesthetic agents during physiological experiments, special difficulties arise in ensuring that the animal does not experience unnecessary pain or distress. It is the responsibility of the person conducting the experiment to ensure that anaesthesia is adequate. Muscle relaxants should never be used without anaesthesia.

1. For any experiment using muscle relaxants it should first be established that the proposed anaesthetic regime is adequate, in the absence of relaxants, to provide analgesia for any surgical procedure or noxious stimulus which is proposed. When light levels of anaesthesia are considered appropriate for experimental purposes, it should have been established that deeper levels of anaesthesia would interfere with the purpose of the experiment. All preparatory major surgery should be performed under full surgical anaesthesia. Subsequent procedures under light anaesthesia in the presence of relaxants should be conducted in such a way that any residual pain from the initial surgery is

blocked by local anaesthetics or analgesia and no further noxious stimuli are delivered. It should be noted that some methods of head holding using ear bars and zygomatic bars are a potential source of pain: other, atraumatic, methods of head restraint should be used in lightly anaesthetized animals.

2. During the course of each experiment in the period when muscle relaxants are used there must be a protocol for continuous or regular assessment of adequacy of anaesthesia. The methods of assessment will be as appropriate to the particular anaesthetic and to the particular experiment. For example:

(a) the monitoring of changes in heart rate and blood pressure provides one of the most valuable indices of the level of anaesthesia. Muscle relaxants, in doses which do not reduce blood pressure, do not abolish autonomic cardiovascular reflexes. A preparation in which precipitate cardiovascular responses occur to minor noxious stimuli must be considered too lightly anaesthetized

(b) the electroencephalogram can be monitored and changes in pattern with minor noxious stimuli may be a valuable guide. Thus, many experiments can be conducted while the EEG is in the unaroused state characteristic of moderate anaesthesia. However, some anaesthetics (e.g. chloralose) induce an EEG that cannot readily be interpreted, and the relation between EEG waveform and the suffering of pain is, in any case, not known. It should also be noted that changes in arterial P_{co_2} and the administration of atropine and certain other drugs may alter the relation between the EEG and behavioural state

(c) the state of the pupil can provide a further indication of the level of anaesthesia. Generally, under anaesthesia pupils are constricted, and dilated pupils, or pupils which react rapidly to stimuli, are indicative of inadequate anaesthesia. This is not a suitable test for experiments on the visual system when drugs have been administered which paralyse accommodation and dilate the pupils.

3. Nitrous oxide (N_2O) has effective mood changing (tranquillizing) and analgesic properties and is useful because of the ease and consistency of delivery but, even at the maximum concentration feasible at normal pressures, it is not an adequate anaesthetic for surgery in cats. Caution should be used in relying upon it for maintenance, even when precautions have been taken to avoid noxious stimuli. It becomes especially important to assess the animal's state periodically and to use supplementary agents as required.'

Copyright and reproduction

Authors may make copies of their own papers in this journal, *provided that such copies are for free distribution only: they must not be sold.*

Authors may re-use their own illustrations in other publications appearing under their own name, without seeking permission, provided that the material is properly acknowledged. Permission to reproduce material from the journal, either in the journal or in other publications, will not generally be given to third parties except with the consent of the authors concerned.

Specific permission will *not* be required for photocopying copyright material in the following circumstances.

1. For private study, provided the copying is done by the person requiring its use, or by an employee of the institution to which he/she belongs, without charge beyond the actual cost of copying.

2. For the production of multiple copies of such material, to be used for *bona fide* educational purposes, provided this is done by a member of the staff of the university, school or other comparable institution, for distribution without profit to student members of that institution, and provided the copy is made from the original journal.

For all other matters relating to the reproduction of copyright material written application must be made to:

The Physiological Society
Administration Office
PO Box 506
Oxford OX1 3XE
United Kingdom

APPENDICES

Appendix 1: The Role of I_h in Firing Properties Of Cultured Nodose Ganglion Neurons

Tissue damage and inflammation increase excitability of nociceptive primary afferent fibers, but the mechanism by which this occurs is not known. Prostaglandins are substances produced in the inflammatory cascade that cause hyperalgesia through a cAMP-dependent mechanism (Ferreira & Nakamura, 1979; Taiwo, Bjerknes, Goetzl & Levine, 1989; Taiwo & Levine, 1991). Prostaglandin E2 (PGE2) increases the excitability (Handwerker, 1976) and sensitizes small-diameter primary afferents to both innocuous and painful stimuli (Martin, Basbaum, Kwiat, Goetzl & Levine, 1987; Schaible & Schmidt, 1988). Therefore, increased levels of cAMP may initiate PGE2-induced sensitization and/or excitation of primary afferent neurons by modulation of a cAMP-dependent ion current involved in the regulation of firing frequencies. I_h is a hyperpolarization-activated cation current modulated by cAMP (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986). Modulation of I_h by increased levels of cAMP leads to increased excitability in sino-atrial node cells of the heart (Brown & DiFrancesco, 1980; DiFrancesco, 1991) and central neurons (McCormick & Pape, 1990). Therefore, since PGE2 and forskolin augment I_h and opioids inhibit I_h via cAMP-dependent mechanisms, I_h may be involved in sensitization of primary afferents by PGE2 and the transmission of chemical pain. In this study, whole-cell recordings were made in current-clamp to determine the influence of I_h on firing patterns of nodose ganglion neurons.

Methods

Whole-cell recordings were made from nodose neurons in culture as described in Ingram & Williams (1994). Kgluconate internal solution was used in these experiments so that K^+ currents would be unaffected. A current-voltage protocol was run immediately after breaking into the whole-cell mode in voltage-clamp. The recording was then switched to current-clamp mode to determine the resting membrane potential and to elicit action potentials. Depolarizing current pulses (450 ms) were injected into the cell at the resting

membrane potential and the threshold (lowest current at which one action potential was elicited) was noted. Depolarizing current pulses two and three times the threshold current were injected to determine if the cell could fire trains of action potentials. This current-clamp protocol was repeated after 5 minute perfusions of PGE2 (1 μM), forskolin (10 μM), 1,9-dideoxyforskolin (10 μM), and Cs^+ (2 mM). Data was collected on an Axopatch 1D amplifier and filtered at 2 kHz. Currents and potentials were digitized and recorded with pClamp software and analyzed using Axograph (Axon Instruments, Inc.) Statistical significance was determined with unpaired t-tests ($p < 0.05$). Descriptive statistics used were the mean and standard error of the mean.

Results

Experiments were run on 38 nodose neurons (Vr = -51 ± 1 mV). Two very distinct firing patterns were obtained from some cells that expressed I_h (n = 34) versus cells that did not (n = 4; Fig. 1). Currents needed to reach threshold were the same for both populations, but neurons without I_h always responded to current injections above threshold with multiple action potentials (n = 4), while cells with I_h had variable responses. At 2X threshold current injection, 19/34 cells responded with only one action potential. In the other 15 neurons, the stronger depolarization evoked multiple action potentials (10/15 cells had I_hs smaller than 2 nA at 100 mV).

PGE2 (1 μM) was tested in 14 neurons. PGE2 decreased the threshold by 106 ± 70 pA, depolarized the membrane potential by 3 ± 1 mV and produced a small, variable increase in the number of action potentials with the control threshold current in 10/14 neurons (Fig. 2). The four cells that were not affected had an I_h greater than 3 nA at -100 mV (n = 4). PGE2-sensitive cells (n = 10) had smaller I_hs that ranged from 0.5 to 3 nA at -100 mV. Control thresholds were not significantly different between these two populations, but PGE2 significantly decreased the thresholds of cells with I_hs smaller than 3 nA (unpaired ttest, $p < 0.05$).

Forskolin (10 μM) decreased the threshold by 100 ± 42 pA and depolarized the membrane potential by 6 ± 2 mV in 7 neurons tested. In order to determine if the effect of forskolin was via adenylyl cyclase, experiments were performed with 1,9-dideoxyforskolin (10 μM; n = 10). Dideoxyforskolin increased the threshold by 20 ± 20 pA and depolarized the cells by 1 ± 1 mV. Both of these parameters were significantly different between cells given dideoxyforskolin or forskolin (unpaired ttest, $p < 0.05$). However, Cs^+ (2 mM) did not block the effects of forskolin consistently (n = 4). In fact, cells often became more active during perfusion of Cs^+. These results may be due to K^+ channel blockade by forskolin and Cs^+.

Discussion

Although the nodose ganglion can be separated into two major types of primary afferents, C-fibers and Aδ-fibers, it is obvious from these experiments that the classification is much more complex. There are several subpopulations of neurons based solely on firing characteristics and expression of I_h. I_h is augmented by PGE2 and forskolin and inhibited by opioids, indicating that I_h may play a role in excitation of primary afferents during painful states. I_h has been shown to modulate cardiac heart rate (DiFrancesco, Ducouret & Robinson, 1989; Denyer & Brown, 1990) and central neuron firing patterns (McCormick & Pape, 1990; Banks, Pearce & Smith, 1993), but all of these cells fire spontaneously. Since primary afferents do not fire spontaneously, we developed a protocol where long depolarizing current pulses (450 ms) of various strengths were injected to determine the activation threshold. The stimulus was then increased two and three times the threshold strength to attempt to elicit trains of action potentials. Cells that did not express I_h fired trains of action potentials with stimulus strengths just above threshold. On the other hand, neurons with a large I_h (>3 nA at -100 mV) never fired trains of action potentials, even with very large stimuli. PGE2 and forskolin had little to no effect on these two subsets of neurons. The majority of cells, those with an intermediate I_h, fell into a continuum between these two extremes and were modulated by prostaglandin E2 and forskolin.

The effects of PGE2 and forskolin on firing were variable, although both of these substances decreased the threshold and depolarized cells to varying degrees. The effects of forskolin were difficult to interpret for two reasons: 1) forskolin has the ability to block potassium currents in addition to its action on adenylyl cyclase (Hoshi, Garber & Aldrich, 1988), and 2) the best blocker of I_h currently available is Cs^+ (2 mM), also a potassium channel blocker. Dideoxyforskolin, a forskolin analog with no action on adenylyl cyclase, was used to determine if the forskolin effects on excitability might be attributed to a specific increase in cAMP. Statistical analysis showed that forskolin was more effective in decreasing the threshold and depolarizing neurons than dideoxyforskolin. Observations obtained with PGE2 may be easier to interpret because PGE2 activates specific prostaglandin receptors coupled to adenylyl cyclase in primary afferents (Sugimoto, et al., 1994). However, there are reports of prostaglandin receptor isoforms that can couple to other effectors, such as K^+ channels. It is not clear whether these isoforms are present on primary afferent neurons.

In order to confirm the role of I_h in excitability or modulation of excitability, a selective blocker of I_h is needed. The actions of Cs^+ were inconsistent in these studies. Cs^+ application often produced spontaneous activity making it difficult to determine if the effects of forskolin or PGE2 were blocked. These results may be a function of Cs^+ blockade of potassium channels. Thus, Cs^+ is not a good tool to confirm that forskolin or PGE2-induced excitability is via augmentation of I_h, and although our studies show that I_h may have a role in excitability or in determining the threshold for firing, more studies are needed.

References

Banks, M. I., Pearce, R. A. & Smith, P. H. (1993). Hyperpolarization-activated cation current (I_h) in neurons of the medial nucleus of the trapezoid body: voltage-clamp analysis and enhancement by norepinephrine and cAMP suggest a modulatory mechanism in the auditory brain stem. *Journal of Neurophysiology* **70**, 1420-1432.

Brown, H. & DiFrancesco, D. (1980). Voltage-clamp investigations of membrane current underlying pace-maker activity in rabbit sino-atrial node. *Journal of Physiology* **308**, 331-351.

Denyer, J. C. & Brown, H. F. (1990). Pacemaking in rabbit isolated sino-atrial node cells during Cs^+ block of the hyperpolarization-activated current i_f. *Journal of Physiology* **429**, 401-409.

DiFrancesco, D. (1991). The contribution of the 'pacemaker' current (i_f) to generation of spontaneous activity in rabbit sino-atrial node myocytes. *Journal of Physiology* **434**, 23-40.

DiFrancesco, D., Ducouret, P. & Robinson, R. B. (1989). Muscarinic modulation of cardiac rate at low acetylcholine concentrations. *Science* **243**, 669-671.

DiFrancesco, D., Ferroni, A., Mazzanti, M. & Tromba, C. (1986). Properties of the hyperpolarizing-activated current (i_f) in cells isolated from the rabbit sino-atrial node. *Journal of Physiology* **377**, 61-88.

Ferreira, S. H. & Nakamura, M. (1979). I - Prostaglandin hyperalgesia, a $cAMP/Ca^{+2}$ dependent process. *Prostaglandins* **18**, 179-190.

Handwerker, H. O. (1976). Influences of algogenic substances and prostaglandins on the discharges of unmyelinated cutaneous nerve fibers identified as nociceptors. *Advances in Pain Research and Therapy* **1**, 41-45.

Hoshi, T., Garber, S. S. & Aldrich, R. W. (1988). Effect of forskolin on voltage-gated K^+ channels is independent of adenylyl cyclase activation. *Science* **240**, 1652-1654.

Ingram, S. L. & Williams, J. T. (1994). Opioid inhibition of I_h via adenylyl cyclase. *Neuron* **13**, 179-186.

Martin, H. A., Basbaum, A. I., Kwiat, G. C., Goetzl, E. J. & Levine, J. D. (1987). Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-δ mechanonociceptors in the hairy skin of rat hindlimbs. *Neuroscience* **22**, 651-659.

McCormick, D. A. & Pape, H. -C. (1990). Noradrenergic and serotonergic modulation of a hyperpolarization-activated cation current in thalamic relay neurones. *Journal of Physiology* **431**, 319-342.

Schaible, H. -G. & Schmidt, R. F. (1988). Excitation and sensitization of fine articular afferents from cat's knee joint by prostaglandin E_2. *Journal of Physiology* **403**, 91-104.

Sugimoto, Y., Shigemoto, R., Namba, T., Negishi, M., Mizuno, N., Narumiya, S. & Ichikawa, A. (1994). Distribution of the messenger RNA for the prostaglandin E receptor subtype EP_3 in the mouse nervous system. *Neuroscience* **62**, 919-928.

Taiwo, Y. O., Bjerknes, L. K., Goetzl, E. J. & Levine, J. D. (1989). Mediation of primary afferent peripheral hyperalgesia by the cAMP second messenger system. *Neuroscience* **32**, 577-580.

Taiwo, Y. O. & Levine, J. D. (1991). Further confirmation of the role of adenyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neuroscience* **4 4**, 131-135.

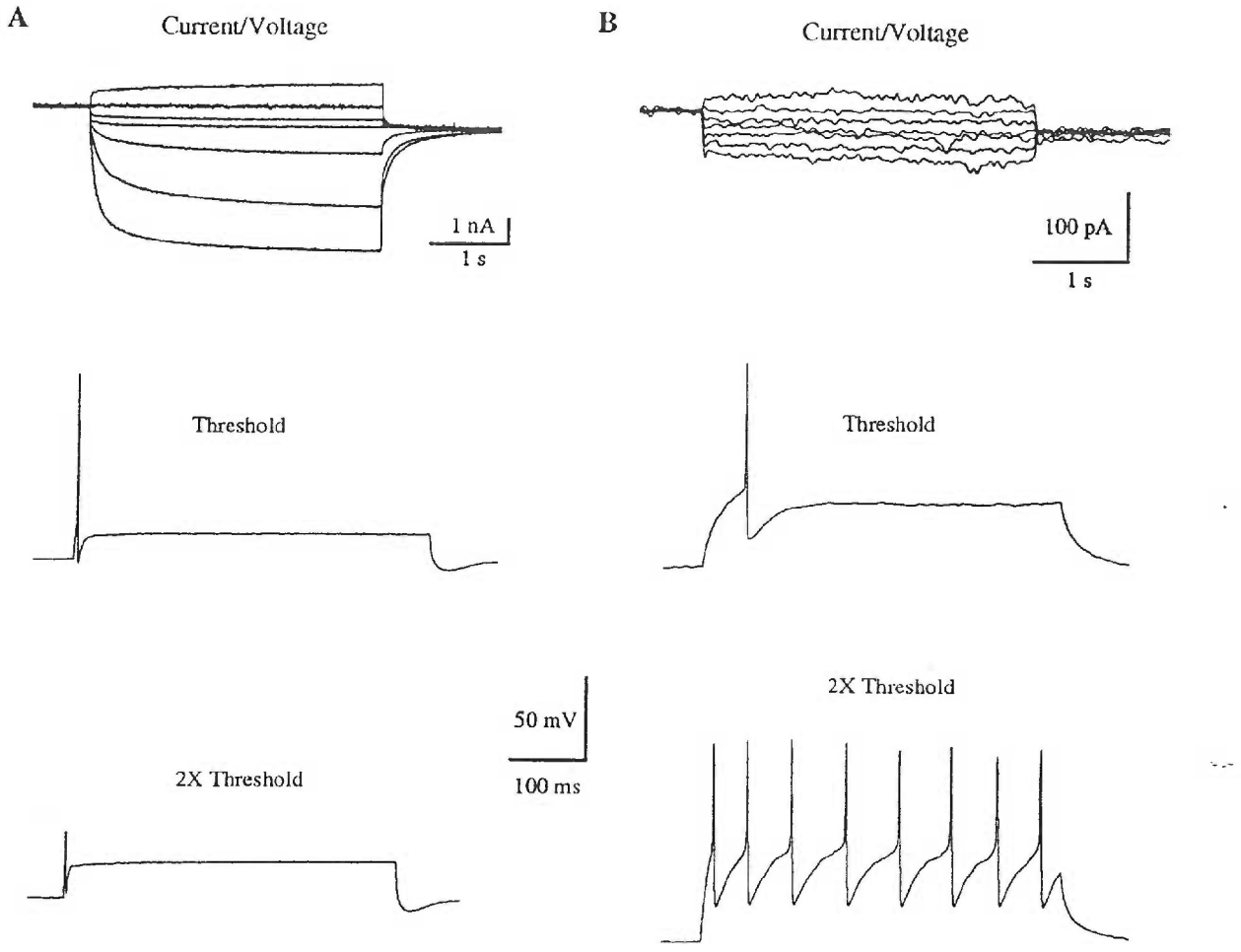

Figure 1. Firing characteristics differ in cells expressing a large I_h current. *A*. Cell expressing a large I_h. Currents evoked by hyperpolarizing voltage steps from Vhold = -40 mV and tail currents elicited at -60 mV. Threshold for AP generation (620 pA at Vr = -58 mV), and only 1 AP is elicited 2X threshold. *B*. Cell that does not express I_h. Currents evoked with same protocol as A (Note change in scale). Threshold (100 pA at -51 mV), and a train of APs is elicited at 2X threshold.

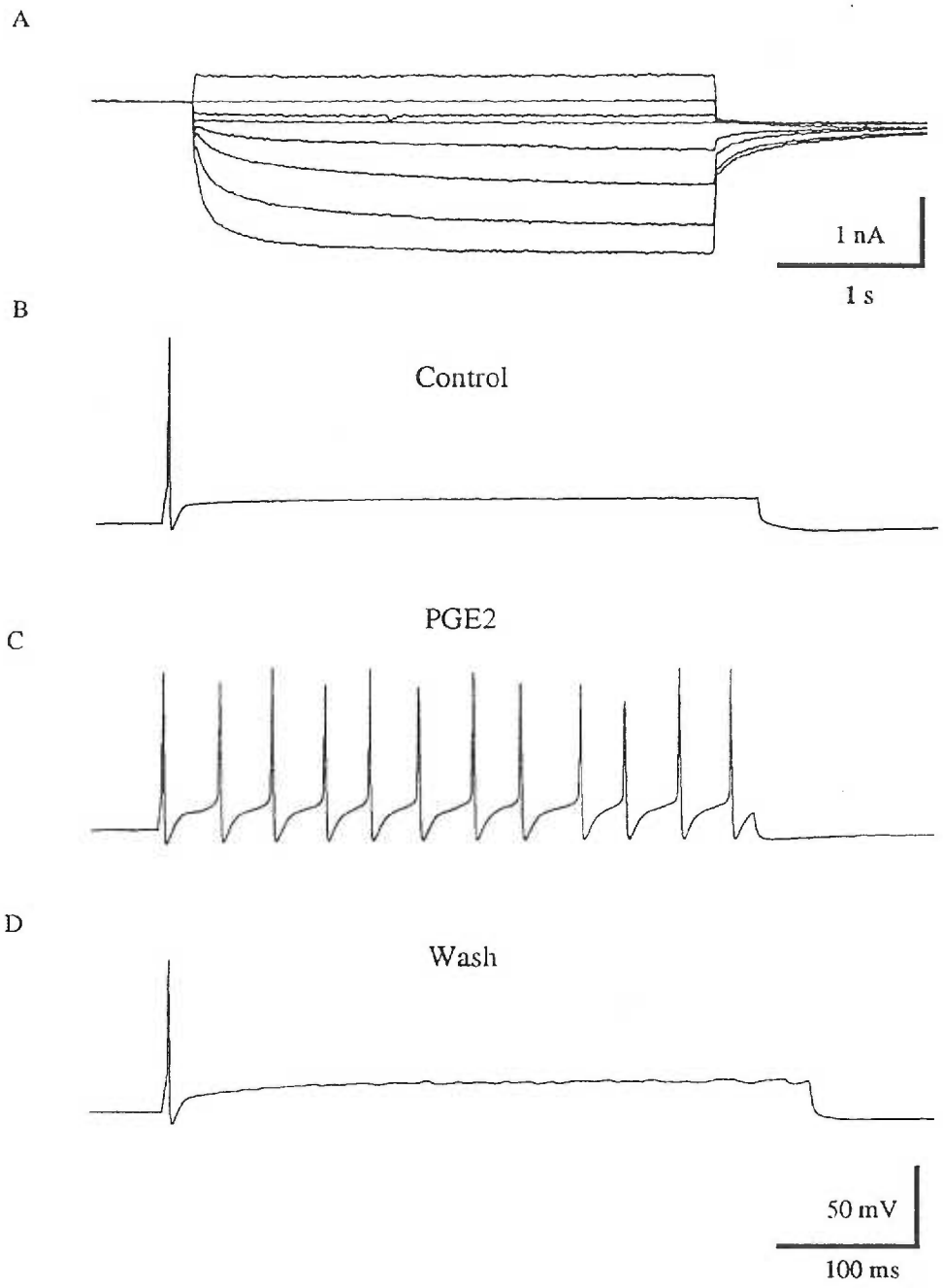

Figure 2. Prostaglandin E2 can decrease threshold and excite nodose ganglion neurons with I_h. *A*, Currents evoked by hyperpolarizing voltage jumps from Vhold = -40 mV, tail currents are elicited with a step back to -60 mV. *B*, One AP is elicited at a threshold of 700 pA, Vr = -55 mV. *C*, Perfusion of PGE2 (1 μM) decreases the threshold to 500 pA, and produces many APs at the control threshold of 700 pA. *D*, Threshold is now 600 pA, but only 1 AP is elicited at 700 pA after a 4 minute wash of PGE2.

Appendix 2: Intracellular recordings from the intact nodose ganglion and dissociated nodose neurons.

Whole-cell recordings from dissociated neurons in culture are a convenient way to study ionic currents in ganglion neurons. However, the technique is associated with potential problems, such as cleaving extracellular portions of membrane receptors and channels during enzyme dissociation. *In vivo*, primary afferent neurons have two processes that bifurcate after leaving the soma extend into the spinal cord and peripheral tissues. The same neurons in culture extend many processes from the soma, indicating a different morphology of the primary afferent neuron. In order to compare characteristics and responses to drugs between dissociated and non-dissociated preparations, intracellular recordings from cells within the intact nodose ganglion were made. In addition, this technique also allowed the determination of I_h characteristics in different subtypes of cells discriminated on the basis of their conduction velocity.

Primary afferent ganglia are composed of heterogeneous cell populations that seem to maintain many of their characteristics in culture (Baccaglini & Hogan, 1983). These populations have been differentiated on the basis of action potential shape, conduction velocities, cell size and responses to drugs (Harper & Lawson, 1985 a, b; McLean, Bennett & Thomas, 1988; Scroggs, Todorovic, Anderson & Fox, 1994). Because opioids only inhibit forskolin-stimulated I_h in a subpopulation of nodose ganglion neurons that could not be identified by morphology, intracellular recordings were also made from nodose neurons in culture to attempt to correlate either cell size or action potential characteristics with the ability of a specific neurons to respond to opioids.

Methods

Intracellular recordings from ganglia:

The nodose ganglia were dissected from adult guinea pigs with a long portion of the vagus nerve attached (approx. 8-10 mm). The ganglion was placed in the recording chamber, and the capsule was opened and pinned. The ganglion was perfused with external solution with the following composition (in mM): NaCl 126; KCl 2.5; MgCl2 1.2; CaCl2 2.4;

NaH2PO4 1.2; NaHCO3 21.4; Glucose 11.1 and gassed with 5%/95% CO/O2 at 37 C. Intracellular recordings were made with 2 M KCl in glass pipettes pulled to 50-80 MOhms. For voltage-clamp recordings, membrane currents were recorded using a single-electrode, voltage-clamp amplifier (Axoclamp 2A) using switching frequencies between 3 and 5 Hz. The switching frequency and capacitance compensation were set at the beginning of each experiment. Settling time of the clamp following a 10 mV step was typically 3-5 ms. Drugs were dissolved into external solution and gravity perfused as previously described.

Action potentials were elicited by injecting a depolarizing current to the nerve within a suction electrode made from a broken glass pipette. Data was collected and digitized by pClamp software and analyzed with AxoGraph (Axon Instruments, Inc.). The conduction velocity was determined by measuring the latency from stimulus to initial action potential rise and dividing by the length of nerve extending from the ganglion to the suction electrode. The action potential duration was measured 20 mV positive to the initial rise of the action potential. The after-hyperpolarization amplitude was measured from the baseline membrane potential to the peak after-hyperpolarization. The decay time constant was determined by an exponential fit of the return of the after-hyperpolarization to baseline (from the peak). Descriptive statistics used were the mean and standard error of the mean. Statistical significance was determined with one-way Anovas ($p < 0.05$) and post-hoc comparisons were made with the Scheffe test ($p < 0.05$).

Intracellular recordings from culture:

Cultures were made and perfused with the external solution described previously (Ingram & Williams, 1994). Intracellular recordings were made as described above. Action potentials were elicited in current-clamp with an injection of current through the pipette to the cell soma. Currents were elicited in voltage-clamp mode by holding the neurons just positive to the activation potential of I_h and stepping to hyperpolarized potentials in 10 mV increments and tail currents were elicited by stepping back to -60 mV. Activation curves were made by measuring the amplitude of tail currents, normalizing the currents to the maximum control current and plotting these values versus membrane potential. Boltzmann

fits to the activation curves were done with Kaleidograph software (Synergy, Inc.) to estimate the half-maximal voltage of activation (V1/2), the maximum amplitude, and slope values in both the absence and presence of drugs. Descriptive statistics used are the mean and standard error of the mean.

Results

Intracellular recordings from the intact ganglion preparation were made from 20 cells that were separated into three groups on the basis of their I_h characteristics: no I_h (n = 9), early I_h (activates more positive than -80 mV; n = 6), and late I_h (activates more negative than -80 mV; n = 5). The conduction velocity of the early I_h group (4 ± 1 m/s) was significantly faster than either the late I_h (1 ± 0.3 m/s) or no I_h (1 ± 0.2 m/s) groups (Fig. 1). Cells with no I_h (2.3 ± 0.3 ms) had significantly longer action potential durations than cells with I_h, whether early (1.2 ± 0.1) or late (1.1 ± 0.3). There were no significant differences between groups on other parameters, such as after-hyperpolarization amplitude or decay time constants of the after-hyperpolarization. Although drug perfusion was attempted, no consistent data was obtained during the perfusion of forskolin (10 μM) or $[met]^5$ enkephalin (1 μM).

Intracellular recordings in culture allowed for longer stable recordings during drug applications (n = 23). Only 12 of these cells had an I_h that activated positive to -80 mV (I_h currents that activated more negative than -80 mV gave inconsistent or no responses to forskolin). Forskolin shifted the V1/2 by 8 ± 1 mV and increased the amplitude by 18 ± 7 %, but had no effect on slope values. 5/12 of these cells responded to $[met]^5$ enkephalin (1 μM) with a 9 ± 1 mV shift in the hyperpolarizing direction in the presence of forskolin. $[Met]^5$ enkephalin did not change the maximum amplitude to the same extent as forskolin (5 ± 9%), and had no effect on the slope values. These are very similar results to whole-cell experiments described by Ingram & Williams (1994). Action potentials were evoked in these experiments also, but there was no obvious correlation of action potential parameters (described in Methods) with responses to

[met]^5 enkephalin.

Discussion

Intracellular recordings from an isolated nodose ganglion showed that there were no obvious differences in I_h characteristics from characteristics observed in cultured nodose ganglion neurons. The main difference was that a larger proportion of neurons did not express an I_h current. However, this was not surprising as the nodose ganglion is predominately composed of unmyelinated, C-type fibers (Agostoni, Chinnock, De Burgh Daly & Murray, 1957) which have been shown to lack I_h (Tokimasa, Shiraishi & Akasu, 1990). C-nociceptors are known to have smaller cell bodies and slow conduction velocities (Harper & Lawson, 1985a, b). Results from the present experiments are consistent with these observations; neurons that did not express I_h had very slow conduction velocities (< 2 m/s), and only the larger neurons in dissociated cultures expressed I_h. Neurons that expressed I_h in the intact ganglion preparation had conduction velocities in the Aδ-fiber range.

Because the intact ganglion preparation only allows blind sampling of neurons, experiments on the effects of forskolin and prostaglandin E2 were limited to the small population of Aδ-neurons. Therefore intracellular experiments were made from nodose neurons in culture where Aδ-neurons (the larger cells) could be visually identified. There were no differences in I_h characteristics or the ability of forskolin and [met]^5 enkephalin to modulate I_h. These experiments also confirmed that dilution of intracellular components with the whole-cell recording technique over time had little effect on our observations of I_h characteristics and modulation of I_h by forskolin and [met]^5 enkephalin. Therefore, we are confident that the whole-cell recording technique is a reliable method for studying I_h from cultured nodose ganglion neurons.

References

Agostoni, E., Chinnock, J. E., De Burgh Daly, M. & Murray, J. G. (1957). Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera of the cat. *Journal of Physiology* **135**, 182-205.

Baccaglini, P. I. & Hogan, P. G. (1983). Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proceedings of the National Academy of Science USA* **80**, 594-598.

Harper, A. A. & Lawson, S. N. (1985a). Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. *Journal of Physiology* **359**, 31-46.

Harper, A. A. & Lawson, S. N. (1985b). Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. *Journal of Physiology* **359**, 47-63.

Ingram, S. L. & Williams, J. T. (1994). Opioid inhibition of I_h via adenylyl cyclase. *Neuron* **13**, 179-186.

McLean, M. J., Bennett, P. B. & Thomas, R. M. (1989). Subtypes of dorsal root ganglion neurons based on different inward currents as measured by whole-cell voltage clamp. *Molecular and Cellular Biochemistry* **80**, 95-107.

Scroggs, R. S., Todorovic, S. M., Anderson, E. G. & Fox, A. P. (1994). Variation in I_H, I_{IR}, and I_{LEAK} between acutely isolated adult rat dorsal root ganglion neurons of different size. *Journal of Neurophysiology* **71**, 271-279.

Tokimasa, T., Shiraishi, M. & Akasu, T. (1990). Morphological and electrophysiological properties of C-cells in bullfrog dorsal root ganglia. *Neuroscience Letters* **116**, 304-308.

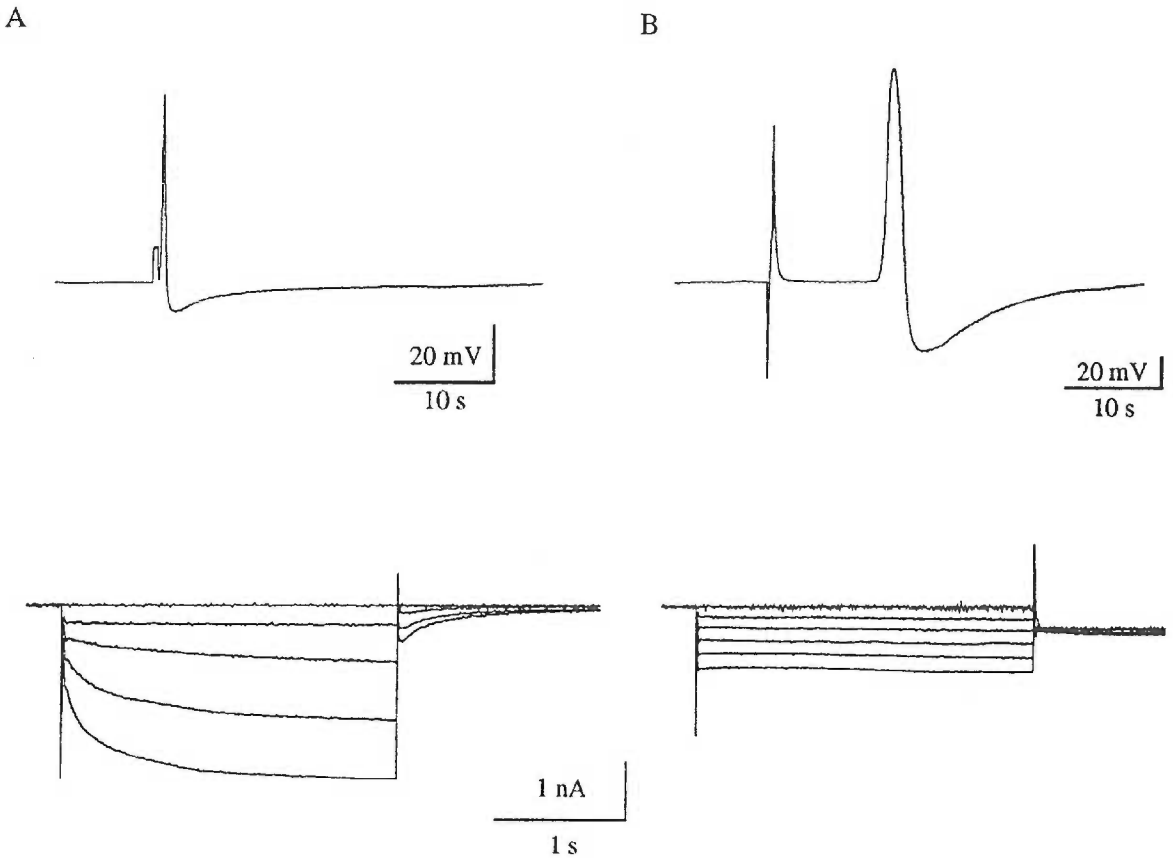

Figure 1. Conduction velocity and AP durations are different in cells expressing I_h. *A*, Intracellular recording from a cell in the nodose ganglion expressing I_h. Top panel: AP evoked by preceding stimulus (stimulus amplitude cut off) in current-clamp mode. C.V. = 6.2 m/s and AP duration = 0.4 ms. Bottom panel: Currents are evoked in voltage-clamp mode by hyperpolarizing voltage steps (10 mV increments for 2.4 s) from Vhold = -40 mV. *B*, Recording from a cell that does not express I_h. Top panel: Stimulated AP: C.V. = 0.7 m/s; AP duration = 3.7 ms. Bottom Panel: Neuron is again held at -40 mV but no I_h is activated with hyperpolarizing steps. The conduction velocities were significantly different overall (Anova, $p < 0.05$) and the Scheffe post-hoc comparison showed significance for the early I_h group versus both the late and no I_h groups ($p < 0.05$). Similarly, the AP duration was significant overall (Anova, $p < 0.05$) and cells with I_h were significantly different than cells not expressing I_h (Scheffe post-hoc test, $p < 0.05$).

Appendix 3: Other Accomplishments

Ingram, S. L., Martenson, M. E. & Baumann, T. K. (1993). Responses of cultured adult monkey trigeminal ganglion neurons to capsaicin. *NeuroReport* **4**,460-462.

Martenson, M. E., Ingram, S. L. & Baumann, T. K. (1994). Potentiation of rabbit trigeminal responses to capsaicin in a low pH environment. *Brain Research* **651**, 143-147.

Baumann, T. K., Burchiel, K. J., Ingram, S. L. & Martenson, M. E. (1995). Responses of adult human dorsal root ganglion neurons in culture to capsaicin and low pH. *Pain, in press.*

CONCLUSIONS

Transduction of Peripheral Pain

It has been known for some time that pain is correlated with excitation of small-diameter primary afferents, however, the mechanisms by which chemicals released during tissue damage cause excitation of these fibers is not known. Hyperalgesia, inflammation and sensitization of nociceptive primary afferents are all associated with increased intracellular cAMP levels, but the function of this increase is not understood. Ion channel activity is the basis for neuronal excitability; therefore, it is probable that ion channels are directly involved in transducing chemical stimuli into excitation of nociceptive primary afferents. The aim of this work was to determine if I_h, a nonselective cation current modulated by cAMP, could be a current involved in the transduction mechanism of inflammatory agents. I_h has been shown to have a role in cAMP-dependent modulation of firing in cardiac myocytes and central neurons. Although I_h was originally described in primary afferents by Mayer & Westbrook (1983), modulation of I_h by cAMP was not demonstrated.

In these studies, we found that a subpopulation of nodose ganglion neurons express I_h. The activation range of $I_h$was between -50 and -80 mV indicating that it may play a role in maintaining the resting membrane potential of these cells. I_h was insensitive to Ba^{+2} and blocked by extracellular Cs^{+2} as previously described in dorsal root ganglion neurons. In general, we observed that the medium to large neurons in dissociated cultures expressed an I_h current while the smallest neurons did not. Because the nodose ganglion consists primarily of Aδ- and C-neurons and C- neurons are the smallest diameter neurons, the medium-large diameter neurons in these cultures were probably Aδ-neurons. In addition, our studies in the intact ganglion preparation determined that cells expressing I_h had conduction velocities in the Aδ-fiber range. Cells with conduction velocities in the C-fiber range usually did not express I_h. These observations are consistent with studies in the dorsal root ganglion that determined that C-neurons do not express I_h (Tokimasa, Shiraishi & Akasu, 1990) and confirmed that I_h is present in a subpopulation of neurons with conduction velocities in the nociceptor fiber range.

Prostaglandins are produced by the inflammatory cascade and cause hyperalgesia and sensitization of small-diameter primary afferents. The exact mechanisms of action of prostaglandin-induced hyperalgesia and sensitization are unknown, but has been shown to be dependent on cAMP. For example, PGE2 has been shown to produce hyperalgesia and increase excitability of primary afferent fibers through a cAMP-dependent mechanism (Taiwo & Levine, 1991; Hingtgen, Waite & Vasko, 1995). The effects of PGE2 on I_h have not been studied previously. Our data demonstrate that PGE2, forskolin and cAMP analogs shift the voltage-dependence and increase the maximum amplitude of I_h in nodose ganglion neurons. These results are similar to those in cardiac myocytes (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986), except that the maximum amplitude is also increased. However, forskolin and PGE2 caused similar increases in I_h maximum amplitudes in sympathetic ganglion neurons (Tokimasa & Akasu, 1990) indicating that there may be differences in modulation of I_h between neurons and other cell types.

The mechanisms by which increased cAMP concentrations cause hyperalgesia and excitation of primary afferents is not known. The inhibitor peptide of protein kinase A has been shown to inhibit PGE2 sensitization of dorsal root ganglion neurons suggesting that protein kinase A may play a role in the increased excitability (Cui & Nicol, 1995; Ouseph, Khasar, & Levine, 1995). Several different mechanisms of I_h modulation by cAMP have been proposed in cardiac myocytes; phosphorylation by PKA, direct stimulation by G-proteins, and direct stimulation by cAMP. Our data demonstrate that cAMP acts directly to modulate I_h. The cAMP analogs, RP-cAMP-S and SP-cAMP-S were used because they are small molecules that can readily diffuse into the cell through the whole-cell pipette. In addition, RP-cAMP-S is an inhibitor and SP-cAMP-S is an activator of protein kinase A. Both analogs shifted that voltage dependence of I_h to more depolarized potentials and occluded the stimulation of I_h by forskolin and PGE2. The inhibitor peptide of protein kinase A, the catalytic subunit of protein kinase A and phosphatase inhibitors had no effect on I_h or forskolin modulation of I_h. These results suggest that cAMP directly modulates I_h in nodose ganglion neurons and that I_h may be functionally related to other cyclic

nucleotide-gated channels in retinal and olfactory sensory neurons. Therefore, direct modulation of I_h by cAMP and phosphorylation of other proteins by PKA may be involved in the sensitizing effects of PGE2.

Modulation of I_h has been shown to regulate firing frequencies in both cardiac myocytes and central neurons. We were interested in determining if I_h also played a role in firing properties of primary afferent neurons. Our data showed that PGE2 and forskolin decrease the threshold of activation, depolarize the membrane and increase the number of action potentials during suprathreshold stimulation in nodose ganglion neurons. Unfortunately, these substances are known to block potassium channels which are important components of excitability. There are no specific I_h blockers to determine if PGE2 and forskolin effects on excitability are a primary result of modulation of I_h. Experiments with dideoxyforskolin, an analog of forskolin that does not activate adenylyl cyclase but has many of the nonspecific effects of forskolin, support a role of I_h in these changes in excitability. In contrast to forskolin, dideoxyforskolin increased the threshold and had no effect on the membrane potential of nodose neurons. In addition, it is clear that I_h is localized to a subpopulation of Aδ-type neurons, is modulated by substances released during tissue damage and inflammation, and has an activation range near the resting membrane potential of primary afferent neurons. Therefore, augmentation of I_h through stimulation of adenylyl cyclase may lead to excitation of primary afferents and result in increased pain.

Opioid Inhibition of Peripheral Pain

Opioids inhibit pain at all levels of the central nervous system. Local injections of opioids can inhibit peripheral pain and excitation of small-diameter primary afferents. The mechanism of opioid inhibition is not known. Opioids are known to have several cellular effects: they can activate potassium channels, inhibit Ca^{+2} channels, inhibit adenylyl cyclase, and inhibit the release of neurotransmitters. In our recordings from nodose ganglion neurons, opioids had no effect on I_h and no effect on the membrane potential in

control conditions. These results are consistent with results in other primary afferents which could not find effects of opioids on resting membrane conductances. However, opioids do inhibit Ca^{+2} channels and forskolin or PGE2-stimulated I_h indicating that opioid effects in the periphery may be dependent on a stimulus. Ca^{+2} channels are electrically stimulated by depolarization associated with action potentials, while I_h is chemically stimulated by PGE2 resulting in an increase in intracellular cAMP concentrations. Opioids were previously shown to inhibit PGE2-induced hyperalgesia and PGE2-increased cAMP levels. Our data has demonstrated for the first time a function (modulation of an ion channel) for opioid inhibition of adenylyl cyclase. μ-receptor agonists, $[met]^5$ enkephalin (ME) and DAMGO inhibited forskolin-stimulated I_h in a dose-dependent manner. The other opioid receptor subtypes (δ- and κ–) had little effect suggesting that opioid inhibition of forskolin-stimulated I_h is mediated by the μ-receptor. The effects of ME and DAMGO were blocked with naloxone suggesting that the inhibition was a selective opioid effect. Opioids had no effect on forskolin-stimulated I_h in the presence of cAMP analogs confirming that opioid inhibition of I_h was a result of inhibition of adenylyl cyclase. Therefore, opioid inhibition of adenylyl cyclase and subsequent inhibition of I_h during inflammation may be a mechanism by which opioids inhibit primary afferent excitability and relieve pain.

Opioid inhibition of peripheral pain is probably a result of many actions. In addition to modulation of I_h, opioids have been shown to decrease the release of neurotransmitters from primary afferents into the spinal cord and inhibit evoked Ca^{+2}-channels. Therefore, peripheral opioids may have pleiotropic effects to produce a stronger inhibitory response. For example, inhibition of excitation via inhibition of I_h and decreased neurotransmitter release by inhibition of Ca^{+2} currents may both contribute to inhibition of inflammatory pain. The pain system is closely associated with other physiological systems, such as the sympathetic system and the immune system. Thus, the actions of PGE2 and opioids seem

to be analogous to the actions of norepinephrine in the heart. Hille (1992) proposed that norepinephrine acts through multiple pathways to produce more efficient stimulation of the heart. Therefore, it is probable that other channels, transporters, and pumps localized to peripheral terminals of primary afferents are also involved in the transduction of chemical pain during inflammation, as well as inhibition of peripheral pain by opioids. We know that PGE2 does not work in isolation to produce pain, but we propose that modulation of I_h may be one mechanism by which chemical information is transduced into excitation of primary afferents. We also propose that opioid inhibition of I_h may be a mechanism of opioid inhibition of peripheral pain. An understanding of molecular mechanisms of pain transduction and opioid inhibition of peripheral pain will be crucial for determining new and better treatments for chronic pain. This is especially important in light of new information concerning complex interactions between the peripheral pain system and other physiological systems.

Future Directions

We have shown that I_h in cultured primary afferent neurons is modulated by PGE2 and opioids, but the physiological role of I_h in primary afferents is not understood. Therefore, in future studies it would be interesting to further pursue the role of I_h in the firing properties of these neurons. These studies could be attempted in *in vitro* preparations such as the neonatal rat spinal cord-tail preparation (Rueff & Dray, 1993) or the rat skin-nerve preparation (Lang, Novak, Reeh & Handwerker, 1990). Substances found in inflammatory exudates, such as PGE2, serotonin and bradykinin, have been shown to excite and sensitize nociceptive afferents in these preparations. It would be interesting to determine if Cs^+ perfusion might block these actions. Cs^+ perfusion might also be effective in the *in vivo* preparation of Schepelmann, Meßlinger, Schaible & Schmidt (1992) that allows recordings to be made from articular afferents from inflamed knee joints. Unfortunately, the fact that Cs^+ also has blocking effects on K^+ channels may preclude the determination of obvious effects that would be attributed to blockade of I_h. Therefore, it is imperative that more selective blockers of I_h are developed. It would also be interesting to

determine if any of the other substances found in inflammatory exudates that have receptors coupled to adenylyl cyclase, such as serotonin and bradykinin, are also effective at modulating I_h.

Another interesting group of studies would be to determine if I_h is actually expressed in the terminals of primary afferent neurons. In order to do these studies I_h would have to be either isolated as a protein or cloned. Many groups have attempted to clone I_h by homology cloning from SA node tissue using the cAMP-binding domains of cAMP-gated nonselective cation channels in olfactory epithelia and retinal neurons. They have only been successful in pulling out other cAMP-gated channels. This strategy is probably not successful for cloning I_h because I_h is a cAMP-modulated channel, not a cAMP-gated channel. Expression cloning of I_h may be the best way to clone I_h with the present strategies. The best tissue for this may be the retina as photoreceptors express the I_h current and the retina has a concentrated population of photoreceptors. Once a clone has been made and the I_h protein can be expressed, antibodies can be generated to some portion of the protein. Thus, I_h could be localized in slices of tissue with immunohistochemical procedures. Furthermore, more detailed analyses of the expressed channel protein may also increase the chances of developing specific blockers for the channel to allow better studies of the physiological role of I_h in primary afferent neurons.

References

Cui, M. & Nicol, G. D. (1995). Cyclic AMP mediates the prostaglandin E2-induced potentiation of bradykinin excitation in rat sensory neurons. *Neuroscience* **66**, 459-466.

DiFrancesco, D., Ferroni, A., Mazzanti, M. & Tromba, C. (1986). Properties of the hyperpolarizing-activated current (i_f) in cells isolated from the rabbit sino-atrial node. *Journal of Physiology* **377**, 61-88.

Hille, B. (1992). Ionic Channels of Excitable Membranes. Second Ed. Sinauer Associates, Inc. Sunderland, Mass.

Hingtgen, C. M., Waite, K. J. & Vasko, M. R. (1995). Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3',5'-cyclic monophosphate transduction cascade. *Journal of Neuroscience* **15**, 5411-5419.

Lang, E., Novak, A., Reeh, P. & Handwerker, H. O. (1990). Chemosensitivity of fine afferents from rat skin *in vitro*. *Journal of Neurophysiology* **63**, 887-901.

Mayer, M. L. & Westbrook, G. L. (1983). A voltage-clamp analysis of inward (anomalous) rectification in mouse spinal sensory ganglion neurones. *Journal of Physiology* **340**, 19-45.

Ouseph, A. K., Khasar, S. G. & Levine, J. D. (1995). Multiple second messenger systems act sequentially to mediate rolipram-induced prolongation of prostaglandin E2-induced mechanical hyperalgesia in the rat. *Neuroscience* **64**, 769-776.

Rueff, A. & Dray, A. (1992). Sensitization of peripheral afferent fibres in the *in vitro* neonatal rat spinal cord-tail by bradykinin and prostaglandins. *Neuroscience* **54**, 527-535.

Schepelmann, K., Meßlinger, K., Schaible, H. -G. & Schmidt, R. F. (1992).

Inflammatory mediators and nociception in the joint: excitation and sensitization of slowly conducting afferent fibers of cat's knee by prostaglandin I2. *Neuroscience* **50**, 237-247.

Taiwo, Y. O. & Levine, J. D. (1991). Further confirmation of the role of adenyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neuroscience* **44**, 131-135.

Tokimasa, T. & Akasu, T. (1990). Cyclic AMP regulates an inward rectifying sodium-potassium current in dissociated bull-frog sympathetic neurones. *Journal of Physiology* **420**, 409- 429.

Tokimasa, T., Shiraishi, M. & Akasu, T. (1990). Morphological and electrophysiological properties of C-cells in bullfrog dorsal root ganglia. *Neuroscience Letters* **116**, 304-308.