PHYSIOLOGY, CHEMISTRY AND IMMUNOCYTOCHEMISTRY OF SELECTED SYNAPTICALLY ELICITED RESPONSES IN DORSAL HORN NEURONES OF THE CAT SPINAL CORD

By

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ABSTRACT

The studies presented in this thesis relate to the physiological, chemical and immunocytochemical characterization of specific synaptically elicited responses following activation of defined sensory inputs to dorsal horn neurones in the spinal cord of the cat.

Low-threshold mechanical stimulation of the skin produced two types of inhibitory postsynaptic potential (IPSP) in dorsal horn neurones. One was shown to involve γ-aminobutyric acid (GABA) acting on a $GABA_A$ receptor and the other one involves adenosine acting on a P_1-purinergic receptor. The adenosine-mediated IPSP is mediated by activation of ATP-sensitive K^+ channels. Further characterization of these IPSPs revealed that the adenosine-mediated IPSP is involved in a system responding optimally to repetitive mechanical stimulation.

Stimulation of hair afferents produced EPSPs which are mediated by an excitatory amino acid. Characterization of the input from single hair afferents to single dorsal horn neurones revealed that the central excitatory response to guard-hair afferent input, but not that to down-hair afferent input is subject to a prolonged $GABA_A$-mediated inhibitory mechanism.

The response to noxious cutaneous stimulation involves at least two types of EPSP in dorsal horn neurones. The fast EPSP in response to a brief stimulus is probably elicited by an excitatory amino acid, whereas sustained noxious stimulation elicits a slow, prolonged EPSP mediated by substance P acting on the NK-1 receptor.

These results demonstrate the participation of specific chemical mechanisms in the mediation of specific physiological responses in identifiable sensory pathways in the spinal cord.

RÉSUMÉ

Les travaux présentés dans cette thèse se rattachent à la caractérisation physiologique, chimique et immunocytochimique de réponses synaptiques évoquées spécifiques suite à l'activation d'entrées sensorielles vers des neurones de la corne dorsale de la moelle épinière chez le chat.

Une stimulation mécanique à seuil-faible de la peau produit deux types de potentiels post-synaptiques inhibiteurs (PPSI) sur les neurones de la corne dorsale. On a montré que l'un de ces potentiels implique la participation de l'acide γ-aminobutyrique agissant sur un récepteur du type $GABA_A$, alors que l'autre PPSI implique la participation de l'adénosine agissant sur un récepteur du type P_1-purinergique. Le PPSI impliquant l'adénosine est dû à l'activation de canaux K^+ ATP-dépendant. Une caractérisation plus poussée de ces PPSI révèle que le PPSI dû à l'adénosine est impliqué dans un système répondant de façon optimale à une stimulation mécanique répétée.

La stimulation d'afférences reliées à des poils donne lieu à des potentiels post-synaptiques excitateurs (PPSE) qui impliquent des acides aminés excitateurs comme médiateurs chimiques. La caractérisation des entrées provenant de fibres afférentes uniques reliées aux poils révèle que la réponse centrale d'excitation à des entrées provenant de poils de garde, mais pas celle provenant de poils de duvet, est soumise à un mécanisme inhibiteur GABAergique impliquant les récepteurs du type $GABA_A$.

La réponse à une stimulation nocive cutanée donne lieu à au moins deux types de PPSE sur les neurones de la corne dorsale. Le PPSE court, en réponse à un stimulus bref, est probablement dû à la libération d'un acide aminé excitateur, alors qu'une stimulation nocive maintenue évoque un PPSE lent et prolongé dont le médiateur chimique est la substance P agissant sur le récepteur du type NK-1. Ces résultats démontrent la participation de mécanismes chimiques spécifiques dans la médiation de réponses physiologiques spécifiques à l'intérieur de voies sensorielles dans la moelle épinière.

PREFACE

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Chapter II:

De Koninck, Y. and Henry, J.L. Peripheral vibration causes an adenosine-mediated postsynaptic inhibitory potential in dorsal horn neurones in the cat spinal cord. (*Submitted for publication*).

Chapter III:

Salter, M.W., De Koninck, Y. and Henry, J.L. Purinergic IPSP in nociceptive dorsal horn neurones is mediated by ATP-sensitive K^+ channels. (*Submitted for publication*).

Chapter IV:

De Koninck, Y., Salter, M.W. and Henry, J.L. Two physiologically and pharmacologically distinct IPSPs in cat dorsal horn neurones elicited by low-threshold mechanical stimulation: evidence for naturally-elicited purine- and GABA-mediated IPSPs (*To be submitted for publication*).

Chapter V:

De Koninck, Y. and Henry, J.L. Prolonged $GABA_A$-mediated inhibition following input from single hair afferents to single dorsal horn neurones in the cat. (*To be submitted for publication*).

Chapter VI:

De Koninck, Y. and Henry, J.L. Substance P-mediated slow EPSP elicited in dorsal horn neurons *in vivo* by noxious stimulation. (*Submitted for publication*).

Chapter VII:

De Koninck, Y., Ribeiro-da-Silva, A., Henry, J.L. and Cuello, A.C. Spinal dorsal horn neurones responding to noxious input with a slow, prolonged EPSP are characterized by the preferential distribution of apposed substance P-containing varicosities. (*To be submitted for publication*).

For the study described in chapter III, the project was elaborated from discussions between Dr. Salter and Myself. I then conducted the entire experimental part of the study with the help of Dr. Salter in the experiments involving the use of Glibenclamide. Part of the data originated from my earlier study (chapter II), in which I had elaborated the intracellular protocol. We then analyzed the data and developed the paper together.

However, as the original hypothesis was proposed by Dr. Salter, he was made first author of the paper.

For the study described in chapter IV, I conducted the entire intracellular part of the study and part of the extracellular experiments. As Dr. Salter had contributed to some of the preliminary experiments leading to this study, his name was included as a co-author of the paper.

For the study described in chapter VII, I conducted the entire electrophysiological part of the study under the supervis.on of Dr. Henry and did the immunocytochemical and ultrastructural analysis under the supervision of Drs. Ribeiro-da-Silva and Cuello.

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INTRODUCTION

I. PRELIMINARY REMARKS

The main objective of the work presented in this thesis is the identification and characterization of chemical mechanisms underlying selected synaptic[1] functions in sensory systems, as we believe that the specificity of function in the central nervous system (CNS) is reflected not only by the specificity of structure, but also of chemistry. That is, the character of specific synaptic events relies not only on the structure of the interconnection between neurones, but also, and to an important extent, on the properties of the underlying chemical mechanism. We choose to work on the dorsal horn of the spinal cord because it represents the first site of integration of the excitatory and inhibitory mechanisms mediating and regulating sensory information and therefore defines a circumscribed system to study responses to defined inputs. It thus serves as a relatively simple model for studying the principles of physiological mechanisms involved in the transfer of information in the central nervous system as a whole.

In the process of identifying the chemical substrate underlying synaptic events, we rely on several criteria to pursue the idea that a given chemical may be involved in a

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[1]Here we refer to synaptic events or transmission in a broad sense, in which chemicals are synthesized and stored in neurones, are released during activity in these neurones and interact directly or through their metabolites with specific receptors on a postsynaptic membrane leading to changes in postsynaptic excitability.

given synaptic function. These include presence, metabolism, release, action and turnover or uptake (for reviews see Werman, 1966; McLennan, 1970; Phillis, 1970; Krnjević, 1974). However, our ultimate goal is to identify exact synaptic events in which such a chemical mechanism is involved and this requires the detailed characterization of a synaptically elicited response, its correlation with the presence and release of that chemical, its equation with the mechanism of action of that chemical and, finally, its specific blockade by an agent which selectively interferes with the activation of the respective receptor, for example with antagonists. The search for this type of evidence has often been limited by the availability of the pharmacological tools for the characterization of synaptically elicited responses and also by the difficulty in studying these responses in preparations preserving intact both the central tissue and its peripheral inputs. With the recent availability of some pharmacological tools and with the improvement of the technological support it has been possible to use *in vivo* preparations to pursue this search.

The series of studies presented here constitutes attempts to characterize selected synaptic responses in the dorsal horn elicited by activation of defined sensory inputs, with the objective of identifying the chemical mechanisms underlying these synaptic events. As the characterized synaptic events were found to be comparable to the known effects of certain putative transmitters applied exogenously, receptor antagonists were used to

confirm the specific involvement of these chemicals in the mediation of the characterized responses. In addition, in Chapter VII, a correlation between the presence of a specific synaptic response in identified neurones and the presence of the transmitter candidate in fibres contacting these neurones has been used to confirm further the direct involvement of this agent in the mediation of the response.

No attempt will be made here to give an exhaustive description of the studies pertaining to the characterization of inputs, outputs or structural and chemical organization of the dorsal horn as it has already extensively been reviewed elsewhere (Willis and Coggeshall, 1978; Brown, 1981; Davidoff, 1983; Yaksh, 1986). Rather, we shall focus on the presentation of work pertaining to the identification of the chemicals involved in the synaptic responses described in the present thesis with a special emphasis on evidence implicating these putative transmitters in specific functional responses, because this is the orientation of our approach. The chemicals that we have focused on are, in the order in which they are presented in the thesis, adenosine, γ-aminobutyric acid (GABA), excitatory amino-acids and substance P. In each case, a reasonable body of information is available on the involvement of these chemicals in mediating specific synaptic responses in the dorsal horn and pharmacological tools (*e.g.* antagonists) are available which can interfere with the characterized, synaptically elicited responses.

II. PERSPECTIVES IN WHICH DIFFERENT SYNAPTIC RESPONSES WERE STUDIED

An adenosine-mediated synaptic response in the dorsal horn

The first proposal of the involvement of a purine in the chemical mediation of synaptic transmission was made by Holton and Holton (1954). They observed that ATP is released into the circulation following electrical stimulation of sensory nerves at intensities which cause antidromic vasodilatation and that this effect is mimicked by intra-arterial injection of ATP (Holton and Holton, 1954; Holton, 1959). These results indicate that ATP is released by the peripheral terminals of sensory fibres and these authors hence suggested that ATP might also be released by the central terminals of these afferents.

The concept that neurones might release a purine as a synaptic transmitter is however really attributable to Burnstock (1972; 1975). Although a large body of evidence has accumulated since that time, supporting the involvement of purines in synaptic transmission, the concept of purinergic transmission in the CNS remains questioned by many (for example, see Phillis, 1990; Dunwiddie, 1990); this may be mainly because a central synaptically elicited response mediated by adenosine or an adenine nucleotide remains to be identified in several systems. Evidence has been presented recently that adenosine may mediate an inhibition of dorsal horn neurones in response to low-threshold

peripheral vibration stimulation (Salter and Henry, 1987). Chapters II and III of this thesis present studies where we have characterized this synaptically-elicited response and identified its mechanisms. We described an IPSP whose characteristics corresponded to reported mechanisms of action of adenosine applied exogenously and, as this IPSP was blocked by an adenosine receptor antagonist, this provided evidence that this synaptic event is mediated by adenosine. As this provided the first evidence of an adenosine-mediated IPSP in the mammalian CNS, it thus provided a critical piece of evidence in favour of the involvement of adenosine as a chemical mediator of synaptic transmission in the CNS.

Here, we shall briefly review the body of information relating to a role of adenosine in the mediation of synaptic responses in the dorsal horn.

Presence

As adenosine may be derived from the extracellular hydrolysis of ATP (Bruns, 1980; Phillis and Wu, 1981), the results pertaining to the presence and release of ATP are relevant to adenosine-mediated mechanisms. Alund and Olson (1979) have reported binding of quinacrine (which binds to ATP) in varicose fibres within the spinal cord, particularly in the substantia gelatinosa. Braas *et al.* (1986) have used an antibody that recognizes adenosine but not adenine nucleotides and found adenosine immunoreactivity specifically in the cytoplasm of perikarya and fibres of neurones in discrete regions of the rat brain. The most extensive immunoreactive staining of fibres and axon terminals was

observed in the brain stem and spinal cord. In particular, intense staining in fibres and terminals of the substantia gelatinosa was observed. However, neither study attempted to determine whether the labelled compounds were located at sites from which they could be released synaptically. Furthermore, because ATP and adenosine are ubiquitous within cells, it is difficult to assess the meaningfulness of these studies.

Binding of transmembrane transport inhibitors

Other groups have examined the binding of adenosine uptake inhibitors such as nitrobenzylthioinosine (NBI). Sites labelled by these agents may represent both uptake and release sites because the nucleoside transport system is bidirectional (Fredholm *et al.*, 1980, 1983; Wohlhueter and Plagemann, 1982). Geiger and Nagy (1985) report binding of [^{3}H]NBI binding in the spinal grey matter which appears especially concentrated in the substantia gelatinosa of the dorsal horn and in the nucleus caudalis of the spinal trigeminal nucleus. Binding is stronger in dorsal roots than in ventral roots, suggesting a specific distribution of the transport mechanism. In addition, binding is decreased by 35% in the dorsal roots but is unchanged in the ventral roots following treatment with capsaicin at doses which selectively destroy unmyelinated afferents (Nagy and van der Kooy, 1983; Nagy *et al.*, 1983). This suggests that these binding sites may be in large part on unmyelinated primary afferents.

Adenosine deaminase

As adenosine deaminase (the enzyme which degrades adenosine to inosine) is thought to play a significant role in the regulation of extracellular adenosine, it has been suggested that neurones containing adenosine deaminase-like immunoreactivity (ADA-LI) might release adenosine as a neurotransmitter (Nagy *et al.*, 1984). There is however no evidence of a direct relationship between adenosine release from nerve terminals and the presence in these terminals of adenosine-deaminase. Its is nevertheless interesting to note that ADA-LI is present in a subpopulation of small diameter dorsal root ganglion neurones (Nagy *et al.*, 1984) and in nerve fibres primarily located in laminae I and II of the dorsal horn (Nagy and Daddona, 1985). As small diameter afferents have been implicated in nociceptive transmission (Bessou and Perl, 1969; Torebjork and Hallin, 1973; Torebjork, 1974; Torebjork and Ochoa, 1980; Konietzny *et al.*, 1981; Torebjork *et al.*, 1984) and as C-fibres (which include nociceptive afferents) terminate mainly in the superficial laminae of the dorsal horn (Sugiura *et al.*, 1986), the distribution of ADA-LI may suggest a possible involvement of adenosine in the integration of central nociceptive responses.

Release

The original hypothesis expressed by Holton and Holton that ATP may be released from primary afferents was based on the observation that ATP is released in the periphery

following stimulation of sensory nerves (Holton and Holton, 1954; Holton, 1959). However, even though they showed that preservation of the intact sensory but not sympathetic nerve was essential for the release, their experimental protocol did not eliminate the possibility that the released ATP may have been from another source intrinsic to the vascular system. More recently, calcium dependent release of ATP (White *et al.*, 1985), using a luciferin luciferase assay, and of adenosine (Sweeney *et al.*, 1989) has been demonstrated from synaptosomal preparations of the dorsal portion of the cord by depolarizations induced by potassium. Release of both ATP (White *et al.*, 1985) and adenosine (Sweeney *et al.*, 1989) from the dorsal portion of the cord was greater than from the ventral portion. Both adenosine and ATP appeared to be released as a portion but not all of the adenosine measured (Sweeney *et al.*, 1989) is reduced in the presence of 5'-nucleotidase inhibitors (a greater decrease would be expected if the major source of adenosine was through the extracellular hydrolysis of ATP).

A morphine-induced release of adenosine has also been reported (Jurna, 1981; DeLander and Hopkins, 1986; Sweeney *et al.*, 1987a). This release is blocked by naloxone, showing that it is due to an opioid receptor mediated event. In this case, adenosine itself seems to be released, as the level of adenosine measured is unchanged in the presence of 5'-nucleotidase (Sweeney *et al.*, 1987b). This release was abolished following neonatal treatment with capsaicin suggesting that it was originating from small diameter afferents. As some of the released adenosine appears to be originating from the

release of ATP upon other treatments than administration of morphine (Sweeney *et al.*, 1987a, 1988), it thus suggests the existence of at least two pools of releasable purines (*e.g.* ATP may be released from a different class of afferents than those implicated in the morphine-induced release of adenosine) (Sweeney *et al.*, 1989).

Binding

Two main receptors have been described for adenosine, A_1 and A_2. Binding studies have utilized [^{3}H]cyclohexyladenosine ([^{3}H]CHA) for the identification of A_1 receptor sites. For the A_2 receptor sites, because of the lack of a specific A_2 ligand, such studies have used the subtraction of the binding of [^{3}H]CHA or [^{3}H]-N^6-[(*R*)-1-methyl-2-phenylethyl]adenosine(which bind more specifically to A_1 receptors) from that of [^{3}H]5'-*N*-ethylcarboxamido ([^{3}H]NECA) (which binds to both A_1 and A_2 receptors). Stronger binding in the dorsal than in the ventral portion of the spinal cord has been reported (Bruns *et al.*, 1986; Choca *et al.*, 1987) suggesting a specific distribution of binding sites. Binding to both A_1 and A_2 receptors is strongest in the superficial layers of the dorsal horn and in lamina X while it is weaker in the remainder of the spinal cord (Geiger *et al.*, 1984; Choca *et al.*, 1988). Bruns *et al.* (1986) reported stronger A_1 binding than A_2 binding in the dorsal horn, but Choca *et al.* (1987) obtained comparable values for both. Part of the discrepancy may be due to the fact that the two groups obtained different values of specific binding for the A_1 receptor. Binding in the dorsal horn is not changed

after spinal cord transection or dorsal rhizotomy (Geiger *et al.*, 1984; Choca *et al.*, 1988), suggesting weak if any binding on descending fibres or on primary afferents. Lesions of the dorsal horn by injection of kainic acid (which causes neuronal loss and glial proliferation, but does not affect fibres *en passant*; Coyle *et al.*, 1981), significantly decreased the binding suggesting that the receptors are on neurones intrinsic to the dorsal horn.

Electrophysiological effects - in vitro

Presynaptic - Adenosine has a receptor-mediated hyperpolarizing action on both dorsal and ventral root potentials in the isolated toad spinal cord (Phillis and Kirkpatrick, 1978). Based on the fact that the hyperpolarizations were blocked in low Ca^{2+}/high Mg^{2+} perfusing solutions, it was concluded that the effects of adenosine were due to a presynaptic inhibition of transmitter release from primary afferent terminals. Stone (1980) did not observe any effect of adenosine on terminal excitability and suggested that if adenosine causes a presynaptic inhibition in these systems, it probably does so through interference with excitation-secretion coupling directly rather than by changing terminal membrane potential. In cultured dorsal root ganglion neurones, adenosine appears to cause a reduction of calcium currents (Dolphin *et al.*, 1986; Macdonald *et al.*, 1986). This effect of adenosine is receptor mediated (Dolphin *et al.*, 1986), but the receptor activated does not seem to have the typical pharmacological properties of either the A_1

or the A_2 receptor suggesting that it acts perhaps on another putative receptor type (Ribeiro and Sebastiao, 1986). If these effects of adenosine on the soma of the primary afferents reflects what occurs at their central terminals, this could provide a mechanism for the presynaptic inhibitory action of adenosine. Presynaptic inhibitory actions of adenosine have been described in other regions of the CNS, mainly in the hippocampus (Ribeiro and Sebastiao, 1986; Allgaier *et al.*, 1987; Fredholm and Lindgren, 1987; Proctor and Dunwiddie, 1987; Duner Engstrom and Fredholm, 1988; Feuerstein *et al.*, 1988; Deckert and Jorgensen, 1988; Dunwiddie and Fredholm, 1989; Fredholm, 1990).

Postsynaptic - The postsynaptic mechanism of action of adenosine in the CNS have been studied mainly in the hippocampus (Segal, 1982; Greene and Haas, 1985; Proctor and Dunwiddie, 1987; Trussell and Jackson, 1987; Spuler and Grafe, 1989; Gerber *et al.*, 1989). These studies describe a hyperpolarization associated with an increase in membrane conductance and extrapolated reversal potentials in the -85 to -90 mV range, suggesting an increase in a K^+ conductance. Gerber *et al.* (1989) report that adenosine acts through an increase in a voltage- and calcium-insensitive K^+ conductance.

In view of the fact that adenosine has mechanisms of action very similar to those of the $GABA_B$ type, it is important to mention that adenosine receptor antagonists have no effect on $GABA_B$-mediated responses (Perkins and Stone, 1980; Dolphin *et al.*, 1986;

Haas and Greene, 1988) and that they have no effect on the release of GABA (Stone *et al.*, 1981).

Electrophysiological effects - in vivo

The effects of both ATP and AMP have been studied in microiontophoretic studies in the dorsal horn. AMP was used in preference to adenosine in iontophoretic studies because of its greater water solubility. In a recent study, Salter and Henry (1985) examined the effects of ATP and AMP on functionally identified dorsal horn neurones. In their study, ATP was found to have three types of effect on dorsal horn neurones: excitation only, excitation followed by inhibition and inhibition only. AMP was found to have only an inhibitory effect. The inhibitory effect of ATP was attributed to the extracellular hydrolysis of ATP into adenosine because the inhibition, but not the excitation, was blocked by an adenosine receptor antagonist. Such biphasic effects of ATP (with inhibition attributable to its conversion to adenosine) have been reported elsewhere (Phillis and Wu, 1981). Non-nociceptive neurones were only excited by ATP, wide dynamic range neurones (which receive both non-nociceptive and nociceptive input) showed all three kinds of effect and nociceptive specific neurones were only inhibited. As the excitatory effect of ATP appeared associated with neurones receiving non-nociceptive input, it was suggested that if ATP was released from primary afferents it may be from low-threshold (non-nociceptive) afferents. In addition, as the inhibitory

effect of ATP was seen only with neurones receiving nociceptive input, it was suggested that the mechanism responsible for the extracellular hydrolysis of ATP (leading to the inhibition by adenosine) may be specifically associated with nociceptive neurones. The release of ATP upon non-nociceptive afferent activation and its inhibitory action on nociceptive neurones through its hydrolysis into adenosine represent a possible mechanism to accounted for some of the inhibition of nociceptive neurones by non-nociceptive input (Melzack and Wall, 1982). This hypothesis is consistent with the finding that the only effect of ATP on cuneate neurones was excitation and these neurones do not receive nociceptive input (Galindo *et al.*, 1967). Fyffe and Perl (1984) also observed that the excitatory effect of ATP was on neurones in the dorsal horn receiving only non-nociceptive input. They however did not observe inhibitory effects of ATP. One possible explanation of this discrepancy is that if the neurones that they studied had no background firing activity they could not observe any inhibition. In the trigeminal nucleus caudalis, Salt and Hill (1983a) did not observe any deferential effect of ATP, but they did not tabulate the biphasic and inhibitory effects of ATP which might have differentiated between non-nociceptive and nociceptive neurones.

Synaptic responses thought to be mediated by adenosine

Sweeney and Sawynok (1989) have recently reviewed the evidence that adenosine may be involved in the mediation, at the dorsal horn level, of a descending antinociceptive pathway stimulated by opioids.

Consistent with their original hypothesis that adenosine may be responsible for the inhibition of nociceptive neurones by non-nociceptive input, Salter and Henry (1987) reported an adenosine-mediated depression of nociceptive dorsal horn neurones induced by cutaneous vibration.

Following the results of these studies, the question remained, by what mechanism is adenosine producing this inhibition of dorsal horn neurones? Hence to characterize further the mechanism of action of this endogenous adenosine-mediated synaptic response, we carried out the intracellular studies which are described in chapters II and III of this thesis. It was anticipated that by defining further the inhibitory response in dorsal horn neurones to vibration, we could identify a synaptic mechanism implicating adenosine in the integration of sensory input.

GABA-mediated synaptic responses in the dorsal horn

A colossal amount of work has been done that has implicated GABA in the mediation of primary afferent depolarization in the spinal cord (for reviews, Levy, 1977;

Nicoll and Alger, 1979; Nistri, 1983; Davidoff and Hackman, 1985) as a mechanism of presynaptic inhibition. The majority of these studies has focused on slow potentials recorded in dorsal roots following input from the periphery and on excitability of afferent terminals in the dorsal horn. Fewer studies have focused on the functional relevance of this type of presynaptic inhibition on the postsynaptic cells. Of those studies, few have assessed the extent of the participation of GABA. Despite the wide-spread involvement of GABAergic synapses in the CNS, it is interesting to note that very few studies have focused on postsynaptic GABA-mediated inhibition in the dorsal horn. Chapters IV and V of this thesis include the characterization of a GABA-mediated mechanisms in the dorsal horn elicited following low-threshold afferent input. In chapter IV we have characterized GABA-mediated IPSPs in dorsal horn neurones elicited by low-threshold mechanical stimulation. As we had previously described an adenosine-mediated IPSP (chapters I and II) in response to low-threshold vibrational stimulation, these two types of IPSP (adenosine *vs* GABA) are compared to differentiate them functionally. In chapter IV, the interaction between two low-threshold afferent systems serving a similar function is examined. This study illustrates how these two input systems, which are relatively similar in modality, can lead to distinct central responses and how their regulation by GABA-mediated inhibitory mechanisms can influence their functional impact. Here an overview is briefly given of the anatomical substrate underlying the GABAergic system in the dorsal horn.

Presence

Part of the difficulty in identifying the presence of GABA in neurones has been the difficulty in developing an antibody to a single amino acid (Hodgson *et al.*, 1985). Early studies relied on antibodies recognizing the GABA synthesizing enzyme, glutamate decarboxylase (GAD; McLaughlin *et al.*, 1975; Barber *et al.*, 1978). Robert's group has reported that the highest GAD reactivity is found in cell bodies and axons in laminae I-III of the dorsal horn, with progressively lower levels in deeper laminae (McLaughlin *et al.*, 1975; Barber *et al.*, 1978). At the ultrastructural level, they reported synaptic contact with dendrites of motoneurones, dorsal horn neurones and primary afferent axons (Barber *et al.*, 1978; Barber *et al.*, 1982).

Ribeiro-da-Silva and Coimbra (1980) examined the uptake of radiolabelled GABA in the dorsal horn (terminals labelled selectively with exogenous GABA have been shown to correspond to sites of high affinity uptake process; Wilkin *et al.*, 1974). They observed a preferential distribution in laminae I-III, particularly in laminae I-IIo. The labelled cell bodies had morphological features distinct from marginal layer neurones of Waldeyer (Narotzky and Kerr, 1978). The labelled axon terminals formed synaptic contacts with dendrites in laminae I-II and were found to be axon endings of synaptic glomeruli in laminae II-III.

The results of these studies hence provided evidence of an anatomical substrate underlying both pre- and postsynaptic inhibition from GABAergic neurones. Chapters IV and V will present physiological data pertaining to both types of inhibition.

More recently, GABA antisera (Hodgson *et al.*, 1985) have been used. Todd and McKenzie (1989) did a detailed morphological study of the GABA-immunoreactive neurones in the dorsal horn and reported that the labelling is associated with islet cells in lamina II (none of the stalked cells were stained). These results were consistent with the observation that islet cells have presynaptic dendrites but stalked cells do not (Gobel *et al.*, 1980; Todd, 1988). They are also consistent with the proposition that stalked cells are probably excitatory interneurones whereas islet cells would be inhibitory (Gobel, 1978; Bennett *et al.*, 1979, 1980; Price, 1986). These results, however, do not exclude the participation of other possible types of GABAergic interneurones, as a larger number of cells are positively labelled for GABA following colchicine treatment (Barber *et al.*, 1982) and weak but positive staining is observed throughout the dorsal horn. Todd and Lochhead (1990) have identified GABA-like immunoreactivity in peripheral axons and in vesicle-containing dendrites presynaptic to central varicosities of type I glomeruli (Ribeiro-da-Silva and Coimbra, 1982; Todd and Lochhead, 1990). As the central varicosities of type I glomeruli are thought to originate from small diameter afferents, it suggested that these immunoreactive profiles could represent the neuronal substrate for presynaptic inhibition of these types of afferent.

$GABA_A$-mediated responses

Cl^- dependent IPSPs have been reported in dorsal horn neurones (Hongo *et al.*, 1966, 1968). Bicuculline sensitive inhibition of dorsal horn neurones has also been reported in response to low-threshold stimulation of afferent nerves (Game and Lodge, 1975) and stimulation of the dorsal column (presumably activating afferents ascending in the dorsal column; (Duggan and Foong, 1985). As the physiological properties of putative GABA-mediated IPSPs have not been characterized and in view of the fact that we have described an adenosine-mediated IPSP (Chapters II and III) we conducted a study to compare these two types of IPSP to determine if they are related to different functional responses.

$GABA_B$-mediated responses

A number of studies have proposed a role for $GABA_B$ receptor mediated mechanisms in the dorsal horn, but, to our knowledge, there is no synaptic response in the dorsal horn, to date, in which a $GABA_B$-mediated mechanism seems to be involved.

$GABA_A$-mediated responses in the dorsal horn

As mentioned earlier, whereas a number of studies have focused on the responses of ventral horn neurones to repetitive afferent input few studies have used this type of approach to study the central integration of excitatory input to dorsal horn neurones.

Elegant studies from Tapper's group and Brown's group have examined the responses of single dorsal horn neurones to inputs from single afferents (Tapper *et al.*, 1983, 1987; Craig and Tapper, 1985; Brown *et al.*, 1987a, 1987b). These authors have described different types of inhibition of the second of paired inputs from single afferents, but did not attempt to characterize the pharmacology of these inhibitions. In chapter IV, using the same approach, we characterized different types of central $GABA_A$-mediated inhibitory mechanisms resulting from the activation of low-threshold hair afferents.

Excitatory amino acid mediated synaptic responses in the dorsal horn

The recent development of selective excitatory amino acid receptor antagonists has lead to a number of studies allowing better characterization of their involvement in sensory pathways. Although excitatory amino acids are not the main scope of the studies presented in chapters V and VI of this thesis, we shall be referring to synaptic responses that have been shown to involve excitatory amino acids. Hence, the following is a brief review of the information available implicating excitatory amino acids in specific functional pathways.

Salt and Hill conducted early studies on the effect of excitatory amino acid antagonists on responses elicited by stimulation of nociceptive and non-nociceptive afferents (Salt and Hill, 1981, 1983b; Hill and Salt, 1982). Their general finding was that

responses to low-threshold afferent input were antagonized by non-specific excitatory amino acid antagonists, but unaffected by NMDA receptor antagonists, suggesting that these responses were mediated by activation of non-NMDA receptors. They also reported that noxious mechanical, but not the afterdischarge of noxious thermal responses, were antagonized by a non-specific excitatory amino acid receptor antagonist (Salt and Hill, 1983b). More recently, using more selective antagonists Morris (1989) showed that responses of dorsal horn neurones to stimulation of myelinated afferents are blocked by excitatory amino acid receptor antagonists acting at the kainate/quisqualate receptor. Headley *et al.* (1987) studied the effect of systemic and iontophoretic administration of NMDA receptor antagonists on noxious evoked responses. They found that the nociceptive responses of ventral horn but not dorsal horn neurones are attenuated by NMDA receptor antagonists, suggesting that NMDA receptors are involved in the nociceptive responses of ventral horn neurones but not of dorsal horn neurones. These results were confirmed subsequently (King and Thompson, 1989; Dickenson and Sullivan, 1990). It is of interest to note that Jahr and Jessell (1985) observed, in a preparation of dorsal root ganglion and dorsal horn cells in culture, that the monosynaptic EPSPs resulting from synapses between the dorsal root ganglion cells and the dorsal horn cells were unaffected by NMDA receptor antagonists, but were blocked by the non-specific excitatory amino acid receptor antagonist, kynurenic acid. In cultures of DRG and

ventral horn cells, NMDA was more effective at blocking the polysynaptic response recorded from stimulation of DRG cells.

These data invite the following proposal. As the dorsal horn neurones in culture were strongly sensitive to L-glutamate, kainate and quisqualate, but not to aspartate or NMDA, this suggests that the lack of an NMDA component in the monosynaptic EPSP from a single afferent is due to the lack of NMDA receptors on the postsynaptic membrane of dorsal horn neurones. These latter neurones may be releasing aspartate onto neurones further on in the spinal pathway as the polysynaptic response in motoneurones is blocked by NMDA antagonists. EPSPs in response to Aδ and C-fibre activation in spinal cord slices (Schneider and Perl, 1988; Yoshimura and Jessell, 1990) are also antagonized by non-NMDA receptor antagonists, but a small NMDA receptor mediated component has also been observed (Yoshimura and Jessell, 1990). Schneider and Perl (1988) observed that a number of neurones were insensitive to exogenous application of glutamate (particularly in laminae II-V) and hence suggested that perhaps glutamate is participating only in some of the synaptic responses. This may be of interest in view of the previously proposed role of ATP in the mediation of primary afferent responses (Fyffe and Perl, 1984; Salter and Henry, 1985). The same observation of a large number of glutamate-insensitive neurones was made by Jahr and Jessell (1990). However, these glutamate-insensitive neurones often received afferent evoked EPSPs that were blocked by excitatory amino acid antagonists indicating that there was a functional

excitatory amino acid input to them. Jahr and Jessell (1990) suggest, as a possible explanation for this discrepancy, that high uptake mechanisms (Stallcup *et al.*, 1979; Bridges *et al.*, 1987) or rapid desensitization to glutamate (Trussell *et al.*, 1988; Mayer and Vyklicky, 1989) may explain why exogenous application of glutamate fails to produce observable effects in some cases.

Recently, the development of antibodies to glutamate have allowed studies of its localization in primary afferents (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988; Hepler *et al.*, 1988). Of particular interest is the report of the colocalization of glutamate-like immunoreactivity with that of substance P (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988). Colocalization of transmitters raises questions about functional significance. In the case of a *classical* transmitter and a peptide which are both excitatory, one interesting possibility is that they may be released differentially with different types or patterns of activity in the nerve terminal (Hökfelt *et al.*, 1980; Schultzberg *et al.*, 1982; Lundberg and Hökfelt, 1983). This issue will be addressed in chapter VI.

A substance P mediated synaptic response in the dorsal horn

The original proposal by Lembeck (1953) that substance P may be a primary afferent transmitter was based on his observation that the dorsal roots contained more

substance P than the ventral roots. However, it was only after the isolation of the peptide by Chang and Leeman (1970) that studies on the functional role of substance P in sensory pathways emerged. Very soon after the early studies of the localization (Nilsson *et al.*, 1974; Hökfelt *et al.*, 1975a, 1975b; Takahashi and Otsuka, 1975) and actions of substance P (Konishi and Otsuka, 1971, 1974a, 1974b, 1974c; Takahashi *et al.*, 1974; Krnjević and Morris, 1974; Henry *et al.*, 1975) in the CNS, came the proposal that it may be particularly involved in the regulation of nociceptive transmission in the dorsal horn (Henry, 1976). Despite the evidence that a major source of substance P in the dorsal horn was of primary afferent origin (Takahashi and Otsuka, 1975), already in the very early iontophoretic studies (Krnjević and Morris, 1974; Henry *et al.*, 1975) the long time course of action of substance P suggested that it was probably involved in slower synaptic events than the fast transmission from primary afferents which, as presented above, probably involves excitatory amino acids. Hence, in chapters VI and VII of this thesis, studies are described where we have tried to characterize a slow response in dorsal horn neurones following noxious cutaneous stimulation and correlated this response with the mechanism of action (chapter VI) and the presence (chapter VII) of substance P.

A brief review of the available evidence on the participation of substance P in a specific synaptic response elicited by nociceptive input will now be presented.

Presence

The early observations by Hökfelt *et al.* (1975b) of the localization of substance P in small "probably unmyelinated afferents" led these authors to raise the question of whether this suggested a possible role of substance P in nociceptive transmission. This conjecture relies on the fact that unmyelinated afferents and thin myelinated afferents are composed in a large part of nociceptors (Burgess and Perl, 1967; Perl, 1968; Bessou and Perl, 1969; Bessou *et al.*, 1971; Torebjork and Hallin, 1973; Torebjork, 1974; Torebjork and Ochoa, 1980; Konietzny *et al.*, 1981; LaMotte *et al.*, 1984; Torebjork *et al.*, 1984). The general criticism raised against this type of conclusion is that this population of afferents also includes low-threshold afferents such as D-hair (Aδ fibres), low-threshold mechanoreceptive C-fibres and thermoreceptors. The only study that attempted to correlate the presence of substance P preferentially with a certain type of functionally identified primary afferent was carried by Leah *et al.* (1985), but their results were rather inconclusive because a large proportion of the C-fibres studied had unidentifiable receptive fields and because they had too small a sample of Aδ afferents (n = 4; none with substance P-like immunoreactivity) to draw reliable conclusions. Clearly, more detailed studies of the immunocytochemistry of functionally identified afferent neurones are needed.

The other morphological finding that suggested an interaction between substance P and nociceptive transmission is that substance P-like immunoreactivity is located in

regions of the dorsal horn that have been particularly associated with integration of nociceptive information (see Brown, 1981). Once again, one can argue that a direct functional relationship remains hypothetical.

Binding

Binding of substance P is relatively unchanged following spinal cord transection. However, it is increased (reflecting up-regulation) following dorsal rhizotomy suggesting that most of the receptors are located on neurones intrinsic to the dorsal horn and that they are juxtaposed with primary afferent terminals (Quirion *et al.*, 1983; Yashpal *et al.*, 1991).

Active product and related neurokinins

One of the questions that has been raised with respect to peptides such as substance P is whether the active compound is the peptide itself or is a product of its degradation. Bishop *et al.* (1986) have compared the magnitude of central responses in rats to intrathecal administration of putative endogenous mammalian peptides with those of their analogue amphibian peptides. They tested the activity of the peptides after incubation for different periods in cerebrospinal fluid and showed that the mammalian form of the peptides lost their potency much faster than their amphibian analogues (on which the mammalian enzymes are presumably less active), suggesting that the

degradation products of the peptides are not the active compounds. On the other hand, it has been suggested that, at least in some systems, the active peptides may be metabolites of substance P such as the substance P(1-7) and substance P(7-11) fragments and that each of these fragments may play a different role (Skilling *et al.*, 1990; Igwe *et al.*, 1990).

Of the different substance P-related peptides that belong to the family of tachykinins, neurokinin A is found colocalized with substance P in primary afferents (Ogawa *et al.*, 1985; Too *et al.*, 1989). The colocalization of neurokinin A with substance P in primary afferents together with the fact that the same gene encodes for both peptides (Nakanishi, 1986; Harlan *et al.*, 1989; Takeda *et al.*, 1990) may suggest that they both share the same function. Neurokinin A is released upon noxious stimulation, as is substance P (Duggan *et al.*, 1990; Hope *et al.*, 1990). However, Neurokinin A is less potent than substance P in facilitating the nociceptive tail-flick reflex and its excitatory action does not appear to be restricted to nociceptive neurones in the dorsal horn (Salter and Henry, 1991) as was observed with substance P (Henry, 1976; Randić and Miletić, 1977). This suggests that there may be a functional role for neurokinin A distinct from that of substance P.

Physiological studies

It was the finding that substance P excited specifically nociceptive neurones that prompted the hypothesis that it may be involved in the regulation of nociceptive information (Henry, 1976). The early hypothesis was strengthened by a number of subsequent findings. Substance P is released in the dorsal horn upon high but not low intensity electrical stimulation of sensory nerves (Go and Yaksh, 1987; Broding *et al.*, 1987), and upon noxious but not innocuous stimulation of the skin (Duggan *et al.*, 1987, 1988). Slow responses recorded in ventral roots to high intensity electrical stimulation but not the monosynaptic reflex (Akagi *et al.*, 1985; Nussbaumer *et al.*, 1989) as well as the facilitation of the tail-flick reflex following noxious stimulations of the tail (Cridland and Henry, 1988) are blocked by peptide analogues that have antagonistic activity against substance P.

The type of synaptic response in the dorsal horn underlying these nociceptive responses remains unknown. The mechanisms underlying the slow depolarization induced by exogenous application of substance P has been described in motoneurones *in vivo* (Krnjević, 1991), in spinal cord neurones in culture (Nowak and Macdonald, 1981, 1982) and in dorsal horn neurones in spinal cord slices (Murase *et al.*, 1982; Murase and Randić, 1984). The results of these studies have suggested that the depolarization is due to a decrease in a K^+ conductance (probably an M current; Nowak and Macdonald, 1982; Krnjević, 1991), as has been described with other types of neurones (Katayama *et al.*,

1979). More recently, however, Murase *et al.* (1986) have reported that substance P augments a persistent slow inward calcium-sensitive current and Womack *et al.* (1988) have suggested that substance P may be causing the release of calcium from intracellular stores, hence suggesting that substance P may have several modes of action on dorsal horn neurones. Randić's group has also identified a slow EPSP in response to a train of stimuli to dorsal rootlets attached to their spinal cord slice preparation (Urban and Randić, 1984; Randić *et al.*, 1986). This slow EPSP had characteristics similar to those of the action of exogenously applied substance P and is attenuated by a substance P receptor antagonist (Urban and Randić, 1984), and by monoclonal antibodies to substance P (Randić *et al.*, 1986).

In the studies presented in chapters VI and VII of this thesis we have identified and characterized a slow excitatory response in the dorsal horn which represented a good candidate for a response mediated by endogenous substance P. Thus, in chapter VI, we have tested the effects on this response of a novel nonpeptide antagonist which has recently been discovered and which has a high specificity for the substance P (NK-1) receptor (Snider *et al.*, 1991; McLean *et al.*, 1991). We have also, in chapter VII, correlated the presence of this response in specific dorsal horn neurones with the presence of substance P immunoreactive boutons apposed onto these neurones.

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CHAPTER II

PERIPHERAL VIBRATION CAUSES AN ADENOSINE-MEDIATED POSTSYNAPTIC INHIBITORY POTENTIAL IN DORSAL HORN NEURONES IN THE CAT SPINAL CORD.

ABSTRACT

We have previously reported a vibration-induced, adenosine-mediated inhibition of nociceptive dorsal horn neurones in the cat spinal cord. The present study was conducted to investigate the mechanisms of this inhibition. *In vivo* intracellular recording was obtained from dorsal horn neurones in the lower lumbar segments of the anaesthetized cat. Vibration (80-250Hz for 2-3s every 15 to 20s) was applied to the glabrous skin of the toes of the hindfoot using a feedback-controlled mechanical stimulator.

In 32 of 43 neurones tested, vibration produced a pronounced hyperpolarization of the membrane potential. This hyperpolarization peaked at -10mV and decayed throughout the period of the application of vibration. It was associated with a decrease in membrane resistance, had a reversal potential negative to the resting membrane potential and was Cl^- independent, suggesting that it was due to an increase in a K^+ conductance, properties typical of the response to adenosine. This IPSP was unaffected by intravenous administration of bicuculline, strychnine and naloxone but was blocked by iontophoretic administration of 8-sulphophenyltheophylline, a P_1-purinergic receptor antagonist.

These results confirm our previous finding that vibration-induced inhibition of nociceptive dorsal horn neurones is purine mediated and further suggest that this inhibition involves a postsynaptic inhibitory mechanism. Therefore, these data imply a direct postsynaptic action of endogenous adenosine onto dorsal horn neurones.

INTRODUCTION

Relatively few studies have focused on inhibition of dorsal horn neurones from the activation of low threshold primary afferents (*e.g.* Wall and Cronly-Dillon, 1960a; Hongo *et al.* 1966; Hongo *et al.* 1963; Brown *et al.* 1987; Salter and Henry, 1987a, 1988a; De Koninck and Henry, 1990; Jessell and Yoshimura, 1990). In previous extracellular electrophysiological studies, our laboratory has reported a depression of the firing rate of nociceptive dorsal horn neurones to peripheral application of a vibrational stimulus (Salter and Henry, 1990a, 1990b). This depression was specific to nociceptive neurones because non-nociceptive neurones were excited by vibration (Salter and Henry, 1990a). This depression was of particular interest because of the possibility that it would represent part of the neuronal substrate for the analgesia produced by vibration and low intensity electrical stimulation of peripheral nerves in human (Bini *et al.* 1984; Ekblom and Hansson, 1982; Lundeberg *et al.* 1984; Lundeberg, 1983; Melzack and Wall, 1982; Pertovaara, 1979; Price and Dubner, 1977; Price and Mayer, 1975).

Several lines of argument suggest that these types of analgesia involve an adenosine-mediated mechanism. In our previous experiments, both iontophoretic administration of ATP and peripheral vibration had a preferential depressant effect on nociceptive dorsal horn neurones while they had an excitatory effect on non-nociceptive neurones (Salter and Henry, 1985). The depressant action of both ATP and vibration were specifically blocked by P_1-purinergic receptor antagonists such as caffeine and

8-Sulphophenyltheophylline (8-SPT) (Salter and Henry, 1987a), suggesting that the inhibition induced by both ATP and vibration involved an adenosine-mediated mechanism (Salter and Henry, 1987a). In addition, the vibration-induced inhibition was potentiated by the adenosine uptake inhibitor, dipyridamole (Salter and Henry, 1987a). Finally, both the ATP-induced and the vibration-induced inhibition were specifically potentiated by iontophoretic administration of tachykinins (Salter and Henry, 1987b, 1988b).

As ATP has been postulated to be released from primary afferents (Holton and Holton, 1954; Holton, 1959) and as excitatory responses to ATP seemed to be associated with non-nociceptive input (Fyffe and Perl, 1984; Salter and Henry, 1985) it was suggested that extracellular hydrolysis (Bruns, 1980; Salter and Henry, 1985) of ATP released from low-threshold primary afferents may represent the source of adenosine responsible for the depressant action of vibration on nociceptive dorsal horn neurones (Salter and Henry, 1985, 1990a, 1990b).

These results prompted the present study, to question whether the vibration-induced inhibition involves a direct action on dorsal horn neurones and, if so, whether adenosine is the chemical mediator acting on dorsal horn neurones directly to produce a postsynaptic inhibition as opposed to indirectly through the release of another inhibitory agent. Hence, we recorded intracellularly *in vivo* from cat dorsal horn neurones to study the same response to vibration as in our previous extracellular experiments (Salter and Henry, 1987a, 1990a, 1990b). It was anticipated that if a direct postsynaptic action on

dorsal horn neurones was observed, the membrane mechanisms involved could be compared to those of responses to exogenously administered adenosine and with reported mechanisms of action of adenosine on central neurones *in vitro* (Segal, 1982; Greene and Haas, 1985; Kangrga *et al.* 1987; Trussell and Jackson, 1987; Proctor and Dunwiddie, 1987; Spuler and Grafe, 1989; Gerber *et al.* 1989).

Preliminary results of this study have been presented elsewhere (De Koninck and Henry, 1988).

METHODS

Animal preparation

Experiments were done on 35 cats anaesthetized with α-chloralose (60 mg/kg i.v.) or decerebrated at the inter-collicular level after induction with halothane (Fluothane, Hoechst). The left common carotid artery and the left jugular vein were cannulated and a tracheal cannula was inserted. Spinal segments L_5-L_7 were exposed for recording and the spinal cords were transected at the L_1 vertebral level to eliminate the influence of supraspinal structures. Prior to the spinal transection, to minimize spinal shock the L_1 segment was injected with lidocaine hydrochloride (Xylocaine 1%, Astra; 0.1 mL). The exposed spinal cord was covered with a pool of warm mineral oil to prevent cooling and drying.

Arterial pressure was monitored continuously throughout the experiment and, when necessary, was maintained above 80mmHg by intravenous infusion of dextran (Macrodex, Pharmacia; 6% in normal saline) or noradrenaline bitartrate (Levophed, Winthrop; 0.002% in normal saline).

The cats were paralysed with pancuronium bromide (Pavulon, Organon; 1mg/kg i.v.) and ventilated artificially. The effects of the muscle relaxant were allowed to wear off periodically to assure a sufficient level of anaesthesia. An additional dose of α-chloralose (30mg/kg) was given i.v. 7-9 hours after the first injection and beyond that time when required. End tidal CO_2 concentration was monitored continuously using a

Beckman LB2 Medical Gas Analyzer and was maintained between 4.0 and 5.0%. Rectal temperature was maintained at 38°C using a thermistor-controlled servo-mechanism and an infrared bulb.

Recording

Single glass micropipettes were used for the recording (3M KCl, 2M K-Acetate or 2M $K(CH_3SO_4)$; resistances 20-50MΩ). In some experiments, the micropipettes were combined to a multibarrelled micropipette for extracellular iontophoresis. The solutions used in these cases for iontophoresis were: sodium AMP (0.2M, pH 7.0; Sigma), GABA (1M, pH 6.5; Sigma), 8-SPT (20mM in tris-HCl, pH 8.0, Research Biochemicals Inc.) and a control solution (165mM NaCl, pH 6.5). AMP was preferred to adenosine because of its water solubility and net charge which made it easier to apply by iontophoresis and because it has been shown to be converted to adenosine in the extracellular space to act on P_1-purinergic receptors (Bruns, 1980; Phillis *et al.* 1979; Salter and Henry, 1985; Taylor and Stone, 1978). The micropipettes were mounted on a stepping motor drive (Nanostepper). The electrodes were connected via a locally build DC amplifier (PCA-3; electronic workshop, Department of Physiology, McGill University) to a Tektronix 5111 oscilloscope. The amplified raw signal was recorded on magnetic tape using an Instrutech digitizer (VR-100) and a VCR tape recorder. The output of the amplifier was also connected to a Data Translation DT2801-A analogue-to-digital board in a personal

computer for sampling and analysis of selected evoked post-synaptic responses from peripheral natural and electrical stimulation. The software for data sampling and analysis was developed by one of the authors.

Criteria for a good penetration were as follows: a shift in DC potential of at least 40-50mV upon penetration, a recovery from injury spike within a few seconds, a stable resting membrane potential of 50-65mV and spikes with overshoot.

Intravenous administration of antagonists

Antagonists were administered intravenously via the catheter in the external jugular vein. When possible, intravenous administration was preferred to iontophoretic administration to ensure that the antagonist would reach a wide number of its receptor sites. The dosages were as follows: bicuculline hydrochloride, 0.3-1.0 mg/kg (Research Biochemicals Inc.) (De Koninck and Henry, 1990; Duggan and Foong, 1985; Salter and Henry, 1988a); strychnine sulphate, 0.2-0.6 mg/kg (British Drug Houses); naloxone hydrochloride, 0.1-0.5 mg/kg (Narcan, Endo) (Calvillo *et al.* 1974; Calvillo *et al.* 1979). In experiments where antagonists were administered intravenously, the cats were pretreated with hexamethoniumbromide (10mg/kg i.v. every 5 hours; Sigma) to block the changes in blood pressure induced by bicuculline (Hong and Henry, 1990) and strychnine (Hong *et al.* 1989).

Classification of neurones

Each unit was carefully classified according to its responses to natural and electrical peripheral stimulation. In some experiments, the peroneal, tibial and sural nerves were exposed at the level of the ankle ipsilateral to the side of recording for electrical stimulation. The fur on the foot was unevenly cut to leave some hairs but also to expose the skin in places for natural stimulation. The natural stimuli used were: movement of hair, innocuous touch/pressure, noxious pressure of the foot or the paws, noxious pinch of the skin with serrated forceps and noxious radiant heat. According to its responses each unit was classified as non-nociceptive, wide dynamic range, nociceptive specific or proprioceptive. Non-nociceptive neurones responding only to innocuous stimuli, wide dynamic range neurones responding to both innocuous and noxious stimuli and nociceptive specific neurones responding only to noxious stimuli. Both wide dynamic range and nociceptive specific neurones were considered to be nociceptive.

Vibration was applied on the glabrous skin of the toes outside the primary receptive field using a feedback-controlled mechanical stimulator (Chubbuck, 1966). The parameters used were a sine wave with a frequency of above 80Hz, as these were the frequencies at which inhibition was best obtained (Salter and Henry, 1990b), and a duration of 2-3s, as this represented an optimal duration to study the peak of the response and its decay phase (Salter and Henry, 1987a, 1990b).

RESULTS

Forty-three nociceptive neurones are included in this study. Non-nociceptive neurones are not included because the focus is on the inhibitory component of the response to vibration and non-nociceptive neurones are only excited by vibration (Salter and Henry, 1990a). Of these 43 neurones, 32 showed a clear hyperpolarization in response to vibration. The remainder of the neurones that did not show a clear hyperpolarization were not studied further. The hyperpolarization (10mV) peaked 20ms after the onset of vibration and slowly decayed over the course of the 2-3s period of the application of the stimulus. The time course of the hyperpolarizing potential recorded intracellularly is similar to the depression of the discharge of action potentials (Fig. 1; see also the response to vibration in our previous extracellular recordings Salter and Henry, 1987a, 1990a, 1990b). This match was also seen at different holding potentials and resulting firing rates. At high amplitudes of vibration, inhibition could be maintained throughout the application of vibration. In Fig. 1 a vibration of low amplitude was used to emphasize the parallel between the recovery of the rate of firing and the membrane potential. Note the difference in time course between the afterhyperpolarizations following the action potentials and the time course of the inhibition to vibration. The cell shown in Fig. 1 showed, in addition, a brief excitation at the beginning of the response to vibration. This biphasic response to vibration observed with some neurones has been extensively described in a previous paper (Salter and Henry, 1990b) and will not be

discussed here because the focus of the present study is on the inhibitory component of the response to vibration. However, it is interesting to note here that the hyperpolarizing response to vibration always started with an earlier latency than the excitatory component of the response to vibration (not shown).

When the afferent nerves between the site of vibration and the recording site were sectioned the response to vibration was abolished confirming that this prolonged hyperpolarization was not due to a mechanical artefact of the vibration.

Intracellular recording for up to several hours could be obtained and the response to vibration was maintained stable throughout the period of recording. No fading of the response was observed even after 5 hours of recording.

In 5 cases where intracellular current pulse injections were found satisfactory to measure membrane resistance it was observed that the hyperpolarization was associated with a decrease in input resistance. A typical case is illustrated in Fig. 2. This observation suggests that the hyperpolarization is associated with an increase in membrane conductance to an ion with an equilibrium potential more negative than the resting membrane potential and therefore that a postsynaptic mechanism is involved. It is interesting to note that the extrapolated reversal of the IPSP was around -85mV (Fig. 2) which is consistent with previously reported reversal potentials for adenosine-induced hyperpolarization *in vitro* (Segal, 1982; Greene and Haas, 1985; Trussell and Jackson, 1987; Haas and Greene, 1984).

In 8 cases DC current injection was used to determine the effects on the vibration-induced hyperpolarization of shifts in membrane potential. Fig. 3 illustrates that the amplitude of the IPSP was increased with depolarization and decreased with hyperpolarization. The IPSP was not noticeably reversed even with hyperpolarizing currents of -8nA through electrodes containing KCl. This indicates that the synaptically-induced hyperpolarization did not involve an increase in Cl^- conductance but rather an increase in a conductance to K^+.

Six cells were satisfactorily tested with iontophoretic administration of AMP. Of these, three were hyperpolarized in response to AMP administration and one had its background synaptic activity depressed without apparent postsynaptic hyperpolarization. Fig. 4 illustrates the parallel between the effect of iontophoretic administration of AMP and the response to vibration. In Fig. 4A, AMP caused a reversible hyperpolarization of the membrane potential associated with a decrease in firing. The same cell showed the typical hyperpolarization in response to vibration (Fig. 4B). On the other hand, one cell was not hyperpolarized by AMP but nevertheless its firing activity and the background synaptic activity were both depressed by AMP, suggesting that, despite the lack of a direct postsynaptic effect of AMP on this neurone, AMP inhibited either terminals apposed on the cell or, by diffusion, activated receptors on neurones presynaptic to the one illustrated. The latter cell was also not markedly affected by vibration (not shown).

To confirm the chloride insensitivity of both the vibration-induced and AMP-induced hyperpolarizations, they were compared to Cl^- sensitive hyperpolarizations. In Fig. 5A and 5B an IPSP was recorded in a dorsal horn neurone using a KCl filled electrode. This IPSP was obtained in response to electrical stimulation of the tibial nerve, and one can see both fading away of the response with time (Fig.5B) and reversal with negative (Cl^-) current injection (Fig.5A). Fig. 5C emphasizes the difference between the response to iontophoretic application of GABA and that to AMP when recording with a KCl electrode: the $GABA_A$ response causes a depolarization (increase of a Cl^- conductance) while AMP (metabolised to adenosine; Bruns, 1980; Phillis *et al.* 1979; Salter and Henry, 1985; Stone and Taylor, 1980; Taylor and Stone, 1978) acting on P_1-purinergic receptors (Salter and Henry, 1985) maintains its hyperpolarizing action, consistent with the previously reported adenosine-induced increase in K^+ conductance *in vitro* (Segal, 1982; Greene and Haas, 1985; Kangrga *et al.* 1987; Trussell and Jackson, 1987; Lotan *et al.* 1982; Proctor and Dunwiddie, 1987; Segal, 1982; Spuler and Grafe, 1989; Gerber *et al.* 1989). The AMP-induced hyperpolarization was associated with a decrease in input resistance (Fig. 5D). The fact that this hyperpolarization was independent of Cl^- suggests that it involved an increase in a K^+ conductance as seen with vibration (Figs. 2 and 3).

In previous studies, we have shown that the vibration-induced inhibition was unaffected by bicuculline, strychnine and naloxone, but that it was blocked by caffeine

and 8-SPT. The vibration-induced IPSP recorded in the present study was not affected by naloxone (up to 0.5mg/kg i.v.; n=4), bicuculline or strychnine (up to 1.0mg/kg i.v.; n=4) at doses known to block the effect of endogenous release of GABA, glycine and opiates (De Koninck and Henry, 1990; Duggan and Foong, 1985; Henry, 1979; Salter and Henry, 1988a). The effects of bicuculline and strychnine on the blood pressure were prevented by an injection of hexamethoniumbromide (10mg/kg i.v.) prior to the recording (Hong and Henry, 1990; Hong *et al.* 1989). An example of the response before and after the administration of bicuculline is shown on Fig. 6.

Unlike the other antagonists, the effects of caffeine on the blood pressure are not blocked by hexamethonium. Accordingly, it was impossible to maintain intracellular recording after intravenous injection of caffeine, so a water soluble analog of 8-phenyltheophylline, 8-SPT (Daly *et al.* 1985), was used as an antagonist to P_1-purinergic receptors and was applied by iontophoresis. In 3 cells, iontophoretic application of 8-SPT reversibly blocked the IPSP to vibration (Fig. 7) confirming that the IPSP involved the activation of P_1-purinergic receptors and that this response to vibration was the same as that we reported previously in extracellular experiments (Salter and Henry, 1987a, 1990a, 1990b). Note that in traces where the IPSP was attenuated, the AHPs remain larger than the IPSP showing that the attenuation was due to blockade of P_1-purinergic receptors and not to a mere hyperpolarization of the membrane. This is

further confirmed on the graph comparing the size of the IPSP at different holding potentials before and after 8-SPT (Fig. 7).

DISCUSSION

We had previously reported an adenosine-mediated depression of the firing activity of dorsal horn neurones to vibrational stimulation of the skin (Salter and Henry, 1987a, 1990a, 1990b). The results of the present study extend our previous results and demonstrate a direct adenosine-mediated postsynaptic component of the inhibitory response to vibration. The IPSP to vibration is associated with a decrease in membrane resistance (Fig. 2), has a reversal potential negative to the resting membrane potential (-85mV; Figs. 2 and 3), is Cl^- insensitive (Fig. 3 & 5) and is reversibly blocked by 8-SPT, a P_1-purinergic receptor antagonist. These characteristics are similar to the adenosine-mediated effects that we have observed with exogenous administration of adenosine (in the form of AMP), *viz.* decrease in input resistance (Figs. 4) and Cl^- insensitivity (Fig. 5) (Salter and Henry, 1985, 1987a), properties which are consistent with reported actions of adenosine in rat hippocampal slices (Segal, 1982; Greene and Haas, 1985; Proctor and Dunwiddie, 1987; Trussell and Jackson, 1987; Spuler and Grafe, 1989; Gerber *et al.* 1989), frog oocytes (Lotan *et al.* 1982) and rat spinal cord slices (Kangrga *et al.* 1987). It is both the similarity between the mechanisms of action of the responses to vibration and to adenosine and the fact that hyperpolarization to vibration is specifically blocked by an adenosine receptor antagonist, as we demonstrate here, that suggest the participation of endogenous adenosine in mediating the IPSP observed in the dorsal horn following peripheral vibration.

As in our previous extracellular study (Salter and Henry, 1987a), the fact that other antagonists such as bicuculline, strychnine and naloxone (Fig. 6) did not block the response to vibration further confirms that the postsynaptic hyperpolarization in response to vibration does not involve the activation of $GABA_A$, glycine or opiate receptors, but rather a direct action of adenosine on dorsal horn neurones. The lack of effect of naloxone also suggests that the adenosine-mediated inhibition observed in the present study may represent a different system than the previously reported opiate-induced release of adenosine (Sawynok and Sweeney, 1989).

Despite the fact that the vibration-induced IPSP was markedly affected by manipulation of the membrane potential (Fig. 3), the difficulty to reverse the hyperpolarization may suggest an electrotonically distant site of action of adenosine (distal dendrites). However, potassium IPSPs are typically difficult to reverse, due to activation of rectifying currents at hyperpolarized potentials. It nevertheless shows that the IPSP is Cl^- insensitive as the recording with Cl^- electrodes in our system readily reversed Cl^- IPSPs (Fig. 4).

Hyperpolarizations in response to vibration in the dorsal horn had originally been reported by Wall and Cronly-Dillon (1960a) and recently by Brown et al. (1987). However, no attempt had been made in these earlier studies to determine whether it represented a postsynaptic mechanism and through what membrane mechanism. Furthermore, we have not observed the phasic hyperpolarization separated by spikes

reported by Wall and Cronly-Dillon (1960a), but a rather continuous slowly decaying hyperpolarization throughout the application of the vibratory stimulus.

The time course of the hyperpolarization to vibration was similar to the depression we had previously described in earlier extracellular studies (Salter and Henry, 1987a, 1990b) and was parallel to the depression of the firing activity (Fig. 1), suggesting that a major component of the depression may be through a direct hyperpolarization of dorsal horn neurones as opposed to inhibition of synaptic terminals. It cannot be excluded, however, that there may be also a component of the vibration-induced inhibition presynaptic to the neurone recorded from (either on interneurones or directly on terminals apposed onto the neurone recorded from).

There can be several possibilities envisaged for the source of endogenous adenosine responsible for the inhibition of dorsal horn neurones by peripheral vibration. As mentioned earlier, based on the similarity of the effects of iontophoretically applied ATP (Salter and Henry, 1985, 1987b) and the response to vibration (Salter and Henry, 1987b, 1988b, 1990a, 1990b) as well as on the possibility that ATP may be released from low-threshold primary afferents (Fyffe and Perl, 1984; Holton and Holton, 1954), the simplest explanation for the source of adenosine would be through the extracellular hydrolysis of the ATP (Bruns, 1980; Salter and Henry, 1985) released from low-threshold primary afferents. However, other potential sources of endogenous adenosine in the dorsal horn have been suggested (for review, see Sawynok and Sweeney, 1989). The

possible localization of adenosine in capsaicin sensitive small diameter afferents (Nagy and Daddona, 1985) will not be discussed here as it presumably represents a different class of afferents than the ones activated in the present study and probably involve the participation of adenosine in a different system (Sawynok and Sweeney, 1989). The involvement of a *purinergic* interneurone in the response to vibration is another possibility (Braas *et al.* 1986). Based on pure latency criteria, our results do not satisfactorily allow us to decide whether this inhibition involves the participation of an interneurone (see discussion in Salter and Henry 1990b).

The results of our present study in conjunction with those of our previous studies (Salter and Henry, 1985, 1987a, 1990a, 1990b) thus suggest a rôle for adenosine in the dorsal horn of the spinal cord and represent the demonstration of a physiologically elicited postsynaptic response in the mammalian CNS involving the participation of endogenous adenosine or a related compound and its direct action on dorsal horn neurones.

Fig. 1: Response to peripheral vibration. Comparison between the depression of the rate of discharge of the neurone (peristimulus time histogram on the left: average of 100 stimuli) with the change in membrane potential observed with intracellular recording (sample trace on the right). Vibration was applied for a period of 3s at 100Hz on the glabrous skin of the toe at the site indicated by the arrow in the inset. The low-threshold receptive field is represented by the darkened area and the high-threshold pinch receptive field by the greater hatched area including the darkened area. This receptive field is typical of wide dynamic range neurones. Therefore, this response to vibration is the same as that reported in previous extracellular studies in our laboratory (Salter and Henry, 1987a, 1990a, 1990b).

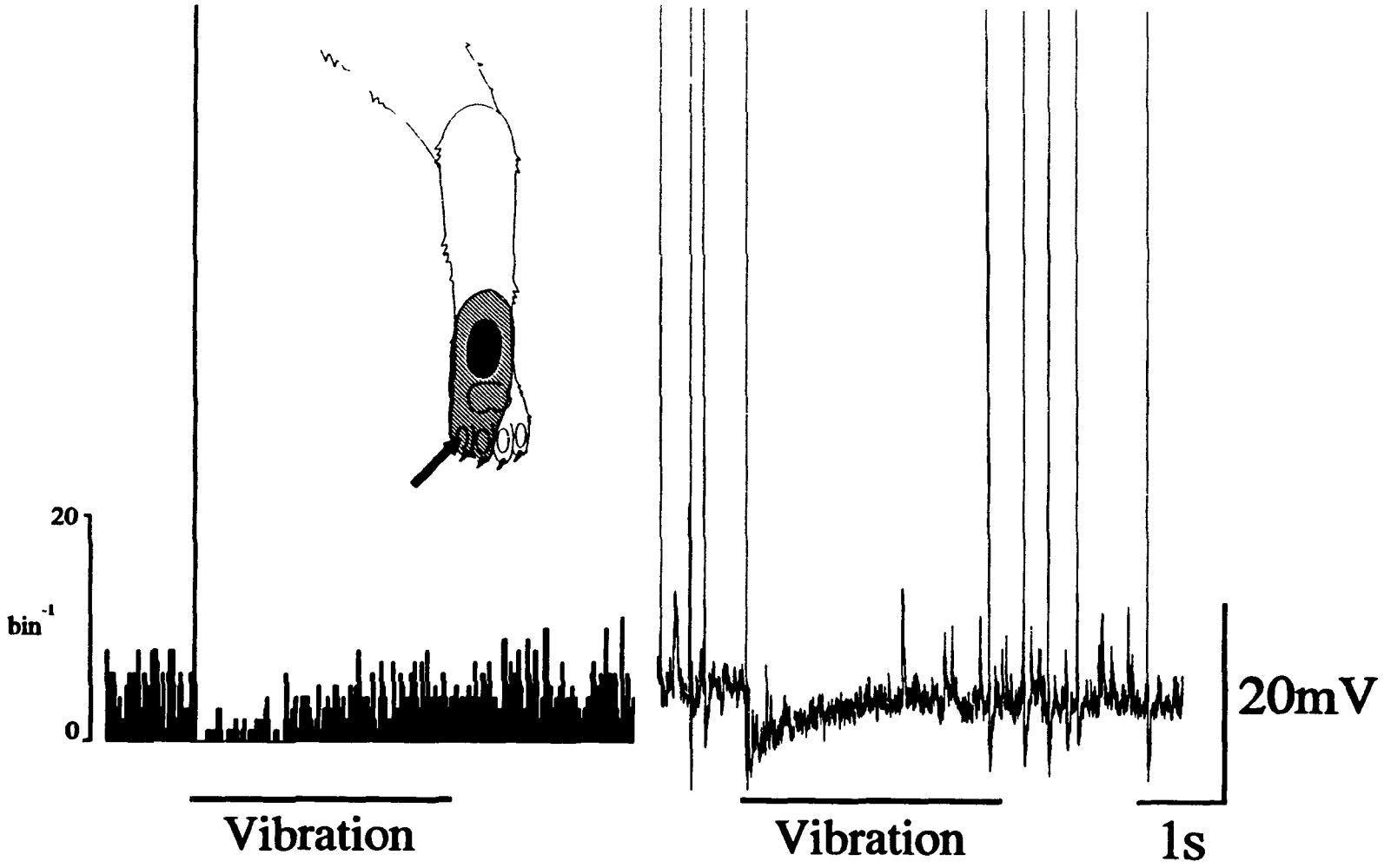

20
bin-1
0
Vibration
Vibration
1s
20mV

Fig. 2: Hyperpolarization associated with a decrease in membrane resistance in a dorsal horn wide dynamic range neurone in response to peripheral vibration (100Hz) applied outside (arrow) the receptive field. **A:** continuous trace showing a typical hyperpolarizing response. The bar below the trace represents the duration of the vibration. **B:** intracellular current pulse injections triggered from the onset of the trace used to compare the membrane resistance before and during the response to vibration. **C:** The two pairs of records illustrate voltage responses to current pulse injections before (left) and during (right) the early phase of the response to vibration. The extrapolated reversal potential was of -84mV as shown on the current voltage relationship (**D**). ● = before vibration, O = during vibration. The schematic diagram in the upper right corner represents the receptive field of the neurone. The darkened area represents the low threshold receptive field and the greater hatched area represents the high-threshold (pinch) receptive field. The arrow points to the location of the vibration probe.

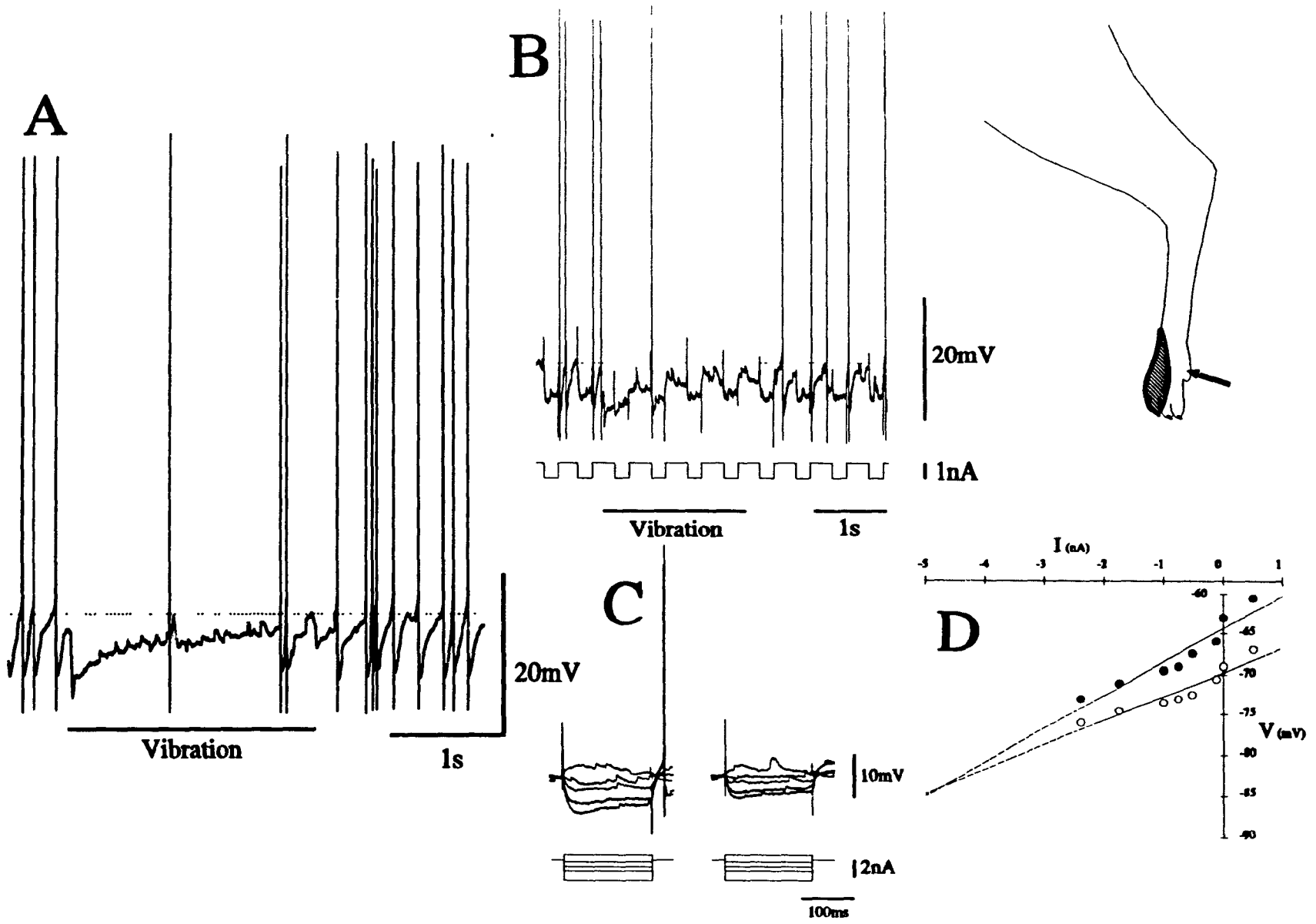

A
20mV
Vibration
1s
B
20mV
1nA
Vibration
1s
C
10mV
2nA
100ms
D
I (nA)
-5
-4
-3
-2
-1
0
1
-60
-65
-70
-75
-80
-85
-90
V (mV)

Fig. 3: Change in the amplitude of the hyperpolarization response to peripheral vibration with a change in the membrane potential. The response increased with depolarization and was abolished with hyperpolarization, but was hardly reversed even with current injection of -6nA. Recording was done with a KCl electrode showing that the response is Cl^- independent. The resting membrane potential was -61mV. Action potentials are truncated for clarity of the illustration. The horizontal bar at the bottom shows the period of application of the vibration stimulation (100Hz). The inset shows a schematic representation of the receptive field of the neurone and of the site of application of vibration (arrow). The darkened area represents the low threshold receptive field and the greater hatched area represents the high-threshold receptive field.

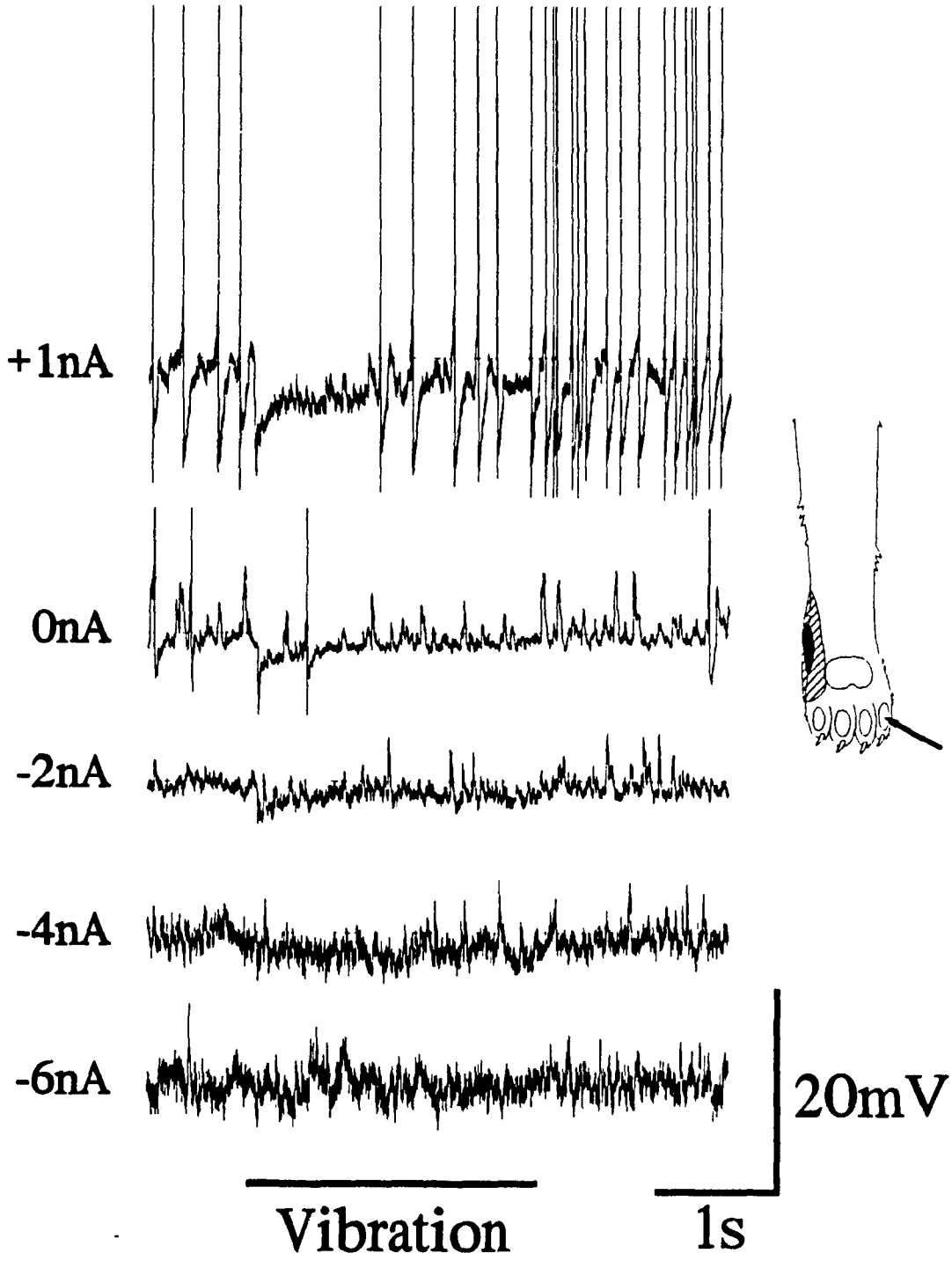

+1nA
0nA
-2nA
-4nA
-6nA
20mV
Vibration
1s

Fig. 4: Comparison between the response to iontophoretically applied AMP and the response to vibration. The neurone in **A** was reversibly hyperpolarized in response to iontophoretic application of AMP. The period of application is represented by the labelled horizontal bar below the intracellular trace. The neurone was unaffected by current as shown by the control current injection through a barrel filled with NaCl (Cl^-). Note the disappearance of afterhyperpolarizations (AHP) during the hyperpolarization to AMP confirming that the change in membrane potential is real. (Resting membrane potential was -65mV). The same neurone showed a hyperpolarizing response to vibration (80Hz) as illustrated by the three superimposed traces on **B**. The receptive field of the neurone is shown on the inset. The darkened area represents the low-threshold receptive field and the larger hatched area the high-threshold pinch receptive field. The vibration probe was placed on the glabrous skin at the location of the arrow. Electrode was filled with $K(CH_3SO_4)$. Currents are in nA.

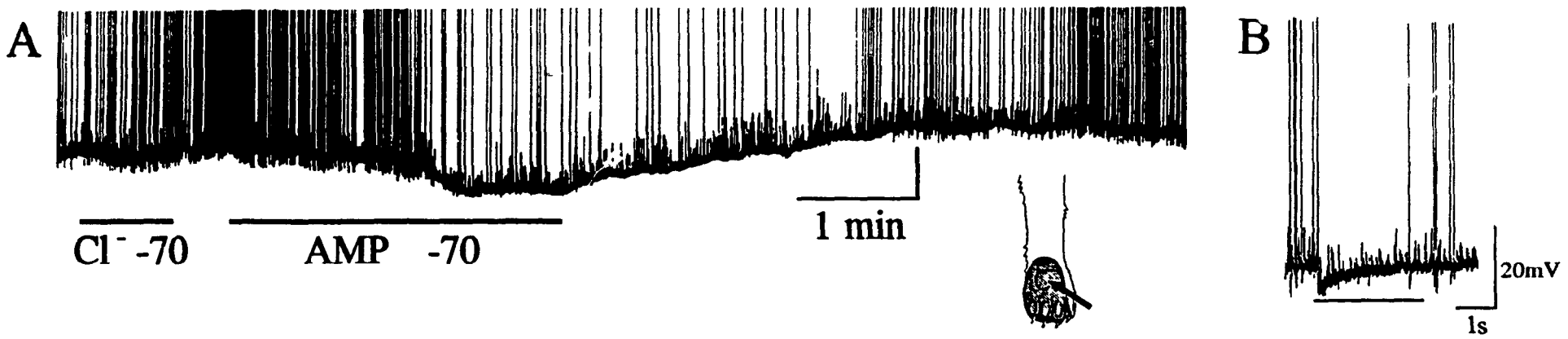

A
Cl⁻ -70
AMP -70
1 min
B
20mV
1s

Fig. 5: Comparison of Cl^- sensitive and Cl^- insensitive responses. **A:** Records are those taken immediately after penetration of the cell with a KCl filled electrode. Cl^- IPSP in response to electrical stimulation of the tibial nerve. Current pulse injections showing reversal of the IPSP at potentials near the resting membrane potential. **B:** fading of the IPSP with time during the recording. Each of the two superimposed traces is an average of 5 IPSPs taken 1 and 2min after the beginning of the recording. **C:** Pen recorder traces showing a comparison of the response to iontophoretic application of GABA (+30nA) and of adenosine 5'-monophosphate (AMP; -30nA) in a different cell using a KCl filled recording electrode. The upward deflections are the *EPSP/reversed IPSP* complex in response to electrical stimulation of the peroneal nerve. The durations of the iontophoretic applications are shown by the horizontal bars below the trace. The response to GABA is a depolarization due to the shift in Cl^- equilibrium potential. On the other hand, the response to AMP is not affected suggesting that it is due to a K^+ conductance. **D:** decrease in membrane resistance (voltage deflections in response to constant current pulse injections) during the hyperpolarization caused by iontophoretic application of AMP (-80nA). Ejection currents are in nA.

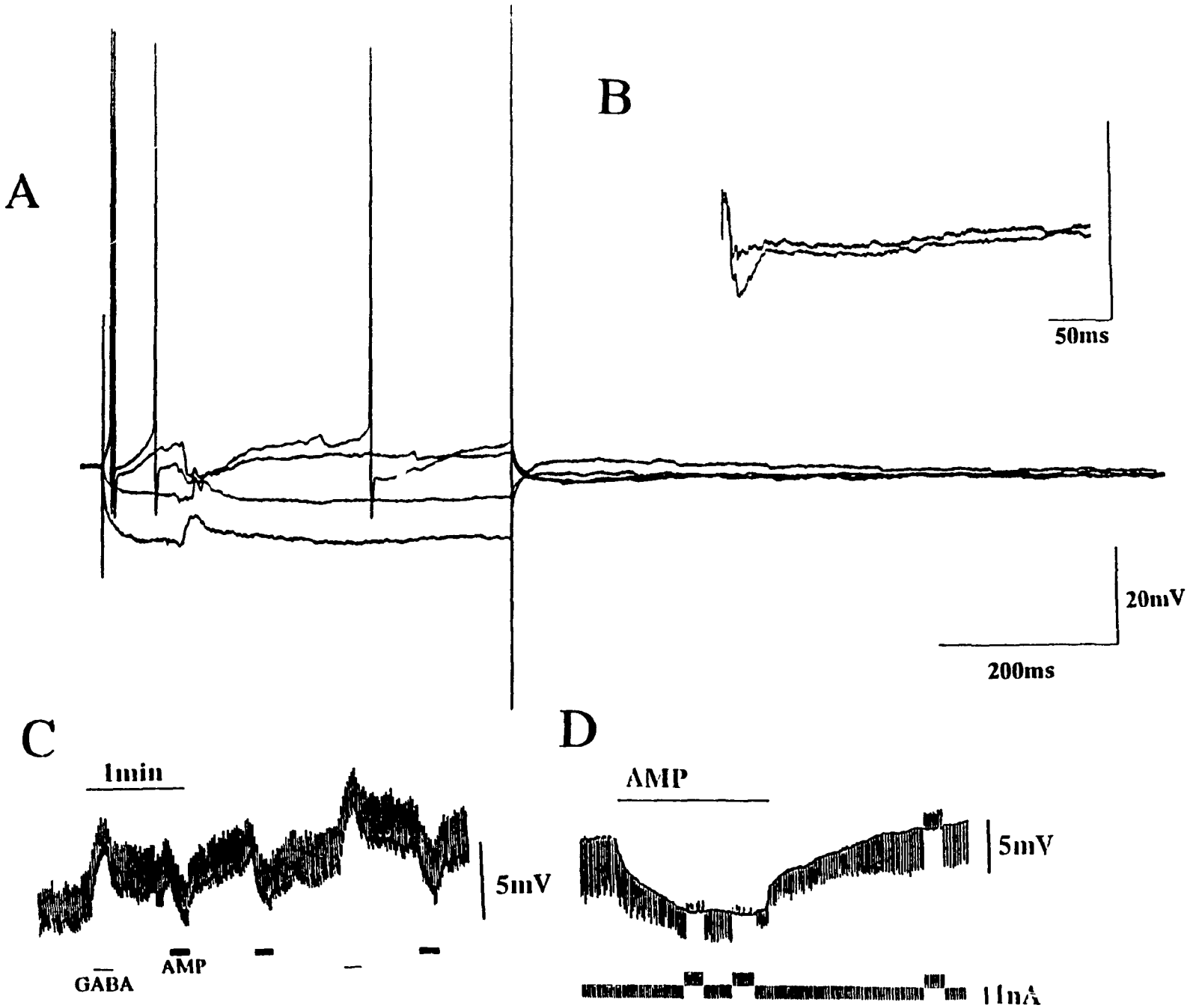

A
B
50ms
20mV
200ms
C
1min
5mV
GABA
AMP
D
AMP
5mV
1nA

Fig. 6: The vibration induced IPSP is not blocked by bicuculline (up to 1mg/kg i.v.). Each set represents 4 superimposed traces showing the response to vibration. Vibration (arrow; 100Hz) was applied for the duration shown by the horizontal bar below the lower trace. Spikes are truncated for clarity of the illustration.

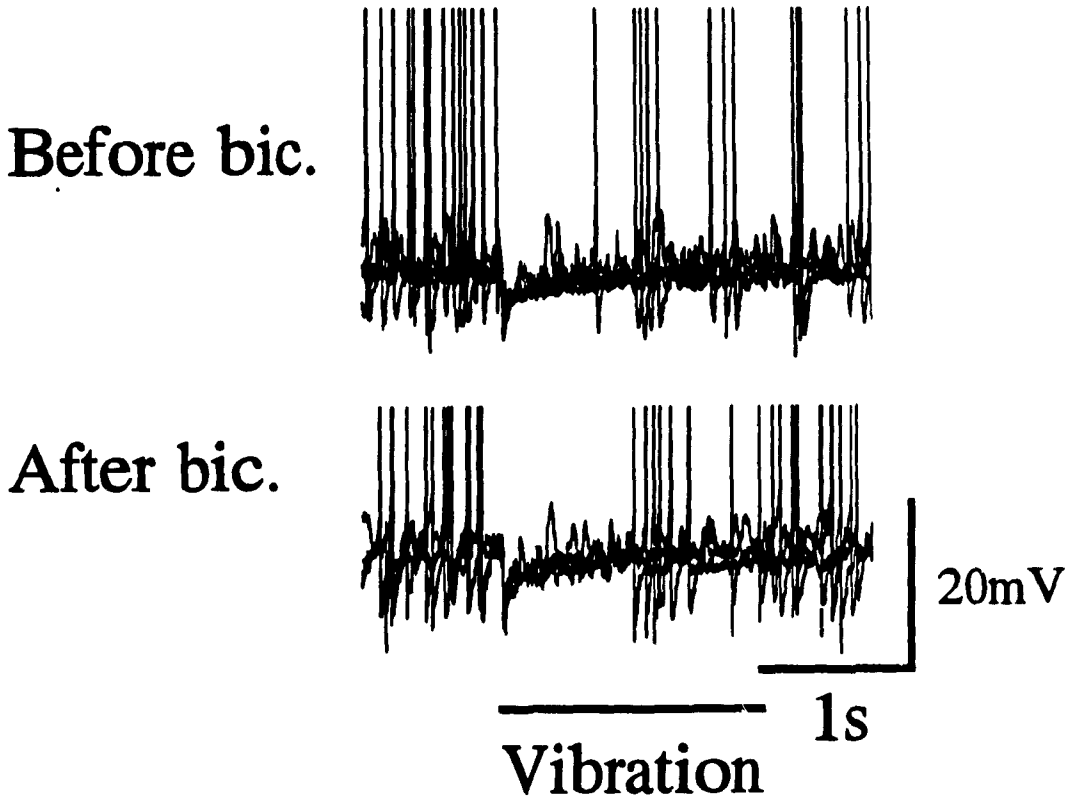
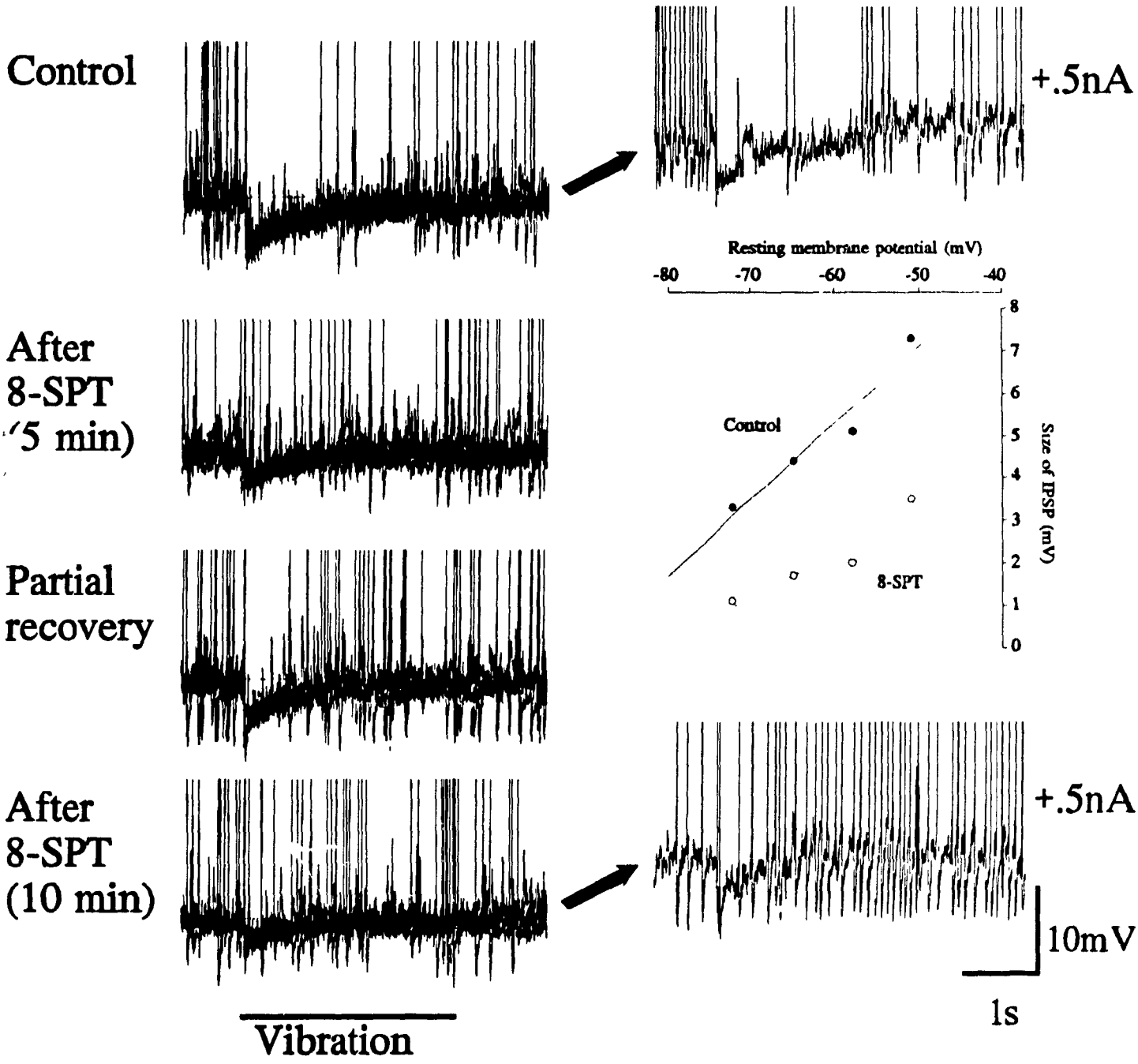

Before bic.
After bic.
20mV
1s
Vibration

Fig. 7 The vibration-induced IPSP is blocked by iontophoretic application of 8-Sulphophenyltheophylline(8-SPT). Each set on the left consists of 5 superimposed consecutive traces showing the response to peripheral vibration. Vibration was applied at 100Hz for a period of 3s as represented by the horizontal bar below the lowest trace. 8-SPT reversibly and repeatably reduced the size of the IPSP in response to vibration.

Note that the afterhyperpolarizations of the action potentials remain larger than the IPSP suggesting that the block of the response is not due to a mere change in membrane potential. This is further emphasized on the right panel: the traces above and below the graph illustrate the response before and after 8-SPT while depolarizing the cell artificially with an intracellular +0.5nA current injection; at a depolarized potential the response to vibration is still dramatically reduced after 8-SPT administration. The graph shows that the size of the IPSP after 8-SPT is smaller at different holding membrane potentials which confirms that the attenuation of the IPSP is due to the blockade of P_1-purinergic receptors and not to a change in membrane potential after 8-SPT application.


Control
+.5nA
Resting membrane potential (mV)
-80
-70
-60
-50
-40
After
8-SPT
(5 min)
Control
8-SPT
Size of IPSP (mV)
0
1
2
3
4
5
6
7
8
Partial
recovery
After
8-SPT
(10 min)
+.5nA
10mV
1s
Vibration

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CHAPTER III

PURINERGIC IPSP IN NOCICEPTIVE DORSAL HORN NEURONES IS MEDIATED BY ATP-SENSITIVE K^+ CHANNELS

Nociceptive dorsal horn neurones, which are involved in the processing of pain-related information, are inhibited by input from vibration-sensitive, large diameter primary sensory fibres (Wall and Cronly-Dillon, 1960a; Salter and Henry, 1990a, 1990b). We have reported previously that the inhibition of spinal nociceptive neurones by vibration is mediated by adenosine acting through P1-purinergic receptors (Salter and Henry, 1987). In a number of different types of cells, adenosine is known to activate K^+ currents (Greene and Haas, 1985; Proctor and Dunwiddie, 1987; Segal, 1982; Trussell and Jackson, 1987; Gerber *et al.*, 1989) and we have recently found that the adenosine-mediated inhibition of nociceptive neurones by vibration is the result of an inhibitory postsynaptic potential (IPSP), which is, indeed, caused by a K^+ conductance (De Koninck and Henry, 1988; chapter II). It has been reported that adenosine-activated K^+ channels in cardiac muscle cells are the ATP-sensitive K^+ channels (Kirsch *et al.*, 1990). Therefore, we questioned whether these channels might mediate the purinergic IPSP we have observed in nociceptive dorsal horn neurones. We report here that glibenclamide, a blocker of ATP-sensitive K^+ channels (Ashcroft, 1988; Schmid Antomarchi *et al.*, 1987a; Schmid Antomarchi *et al.*, 1987b), blocks the inhibition of nociceptive neurones by vibratory stimulation when this compound is administered locally by iontophoresis or systemically by intravenous injection. In addition, direct intracellular injection of ATP

was found to block the IPSP evoked by vibratory stimulation. These data indicate that the purinergic IPSP in nociceptive spinal neurones is mediated via ATP-sensitive K^+ channels. As inhibition of nociceptive dorsal horn neurones may be the physiological basis for the analgesia produced by vibratory stimulation in humans (Bini *et al.*, 1984; Lundeberg, 1984; Lundeberg *et al.*, 1984; Ottoson *et al.*, 1981), we propose that activation of ATP-sensitive K^+ channels in these neurones may mediate the analgesia produced by vibration.

Experiments were done on adult cats anaesthetized with α-chloralose (60 mg/kg I.V. after induction with halothane). In all experiments the spinal cord was transected at the first lumbar level to remove descending influences and to eliminate the possibility that the effects on lumbar dorsal horn neurones might be mediated via supraspinal structures. Extracellular single unit recordings from neurones in spinal segments L_5-L_7 were made using multibarelled micropipettes, described in detail elsewhere (Salter and Henry, 1985). The recording barrel contained 3 M NaCl and other barrels contained sodium L-glutamate (1 M), glibenclamide (10 mM) or the vehicle for glibenclamide which was 150 mM NaCl, 50 mM Tris, 10% dimethylsulfoxide(DMSO). All neurones studied were excited by glutamate, thus eliminating recordings from fibres. Glibenclamide was ejected by passing inward current with respect to the electrode. Intracellular recordings were made using single glass micropipettes filled with 3 M KCl or 3 M $K(CH_3SO_4)$. In some experiments, a multibarrelled micropipette was glued to the intracellular micropipette for

extracellular iontophoresis of 8-sulphophenyltheophylline, a P_1-purinergic receptor antagonist (8-SPT; 20 mM in Tris-HCl, pH 8.0, Research Biochemicals Inc.). Vibratory stimuli were produced by a feedback-controlled mechanical stimulator and the plexiglass probe of the stimulator was placed on the skin 2-4 cm outside the excitatory receptive field. The frequency of vibration was 100 Hz. The stimuli were applied for 3 s and were repeated every 15 s. The site of stimulation and the stimulation amplitude were chosen so as to produce maximum inhibition.

Each of the neurones was tested with natural forms of peripheral stimulation and only those neurones which showed characteristic excitatory responses to noxious stimuli to the hindlimb (Salter and Henry, 1990b) were included. The rate of discharge of more than 95% of nociceptive dorsal horn neurones (n=250) (Salter and Henry, 1987, 1988) is inhibited by vibratory stimulation applied cutaneously. This inhibition is likely mediated by activating Pacinian corpuscle afferents (Salter and Henry, 1990a). All of the neurones included in the present report were inhibited by the vibratory stimulation and stimuli producing maximum inhibition were used in each case. Such inhibitory responses can be evoked reproducibly for periods in excess of 5 hours (Salter and Henry, 1990a).

In order to investigate the possible involvement of ATP-sensitive K^+ channels in the inhibitory response to vibration, a specific blocker of these channels (Ashcroft, 1988; Schmid Antomarchi *et al.*, 1987a; Schmid Antomarchi *et al.*, 1987b), glibenclamide was applied locally by iontophoresis. Glibenclamide reversibly attenuated the inhibition

of firing produced by vibration (Fig. 1). Each application of glibenclamide caused a reproducible and fully reversible attenuation of the inhibitory response. In addition, the degree of attenuation was directly related to the magnitude and duration of the glibenclamide application (Fig. 1B). For all of the neurones tested (n=5) the inhibitory response to vibration was attenuated by iontophoretic application of glibenclamide. In contrast, the inhibition was unaffected by applications of the vehicle for glibenclamide.

These experiments indicate that glibenclamide may act locally within the dorsal horn to attenuate the inhibition evoked by vibratory stimulation. Intravenous administration of glibenclamide (20 mg/kg doses) was also effective in blocking the inhibitory response to vibration (Fig. 2). The effect of glibenclamide peaked 15-20 min after the injection (Fig. 2A) and the extent of recovery in the subsequent 1-2 hours was inversely related to the degree of blockade. In cases where complete recovery was observed, the blockade was found to be fully reproduced when another dose of glibenclamide was given (not shown). In other cases when a single dose of 20 mg/kg did not completely block the inhibition, additional glibenclamide was given just after the effect had peaked (Fig. 2B). This second, cumulative, dose further blocked the inhibition, indicating that the blockade was a dose-dependent effect. On the average, a single dose of 20 mg/kg glibenclamide produced a blockade of 51 $\pm$ 16% (mean $\pm$ SEM, n=5 neurones).

These results imply that activation of ATP-sensitive K^+ channels is necessary to produce the inhibition of firing caused by the vibratory stimuli. We have reported that this inhibition of firing is due to a K^+-dependent IPSP and that this IPSP is caused by adenosine, as it is sensitive to P1-purinergic receptor antagonists such as 8-SPT (Fig. 3D) (De Koninck and Henry, 1988; chapter II). We sought to determine directly whether this IPSP, itself, is mediated by ATP-sensitive K^+ channels. Glibenclamide could not be used to address this point because it is not possible to firmly establish whether the blocker acts directly on the neurone studied with either iontophoretic or intravenous administration. However, as ATP can block the channels from the inside of cells (Ashcroft, 1988), we examined the effect of direct intracellular administration of ATP on the IPSP evoked by vibration. This was done by recording with microelectrodes containing 0.5 M ATP (Tris salt, pH 7.2) and 1.5 M $K(CH_3SO_4)$ and by injecting ATP with hyperpolarizing current. The IPSPs evoked by vibratory stimuli prior to injecting ATP ($n=5$ neurones) were indistinguishable from those recorded with electrodes containing $K(CH_3SO_4)$ ($n = 20$) or KCl ($n=33$) alone (De Koninck and Henry, 1988; chapter II). Although it was not possible to study the IPSP during the injections of ATP (duration 5-10 min) because of membrane hyperpolarization, when the injection ended, the membrane potential returned immediately to the control level and it was found that the IPSP was smaller than the control value. The IPSP subsequently decreased further and the greatest blockade of the IPSP occurred 5-10 min after the end of the ATP injection (Fig. 3B). At this time the

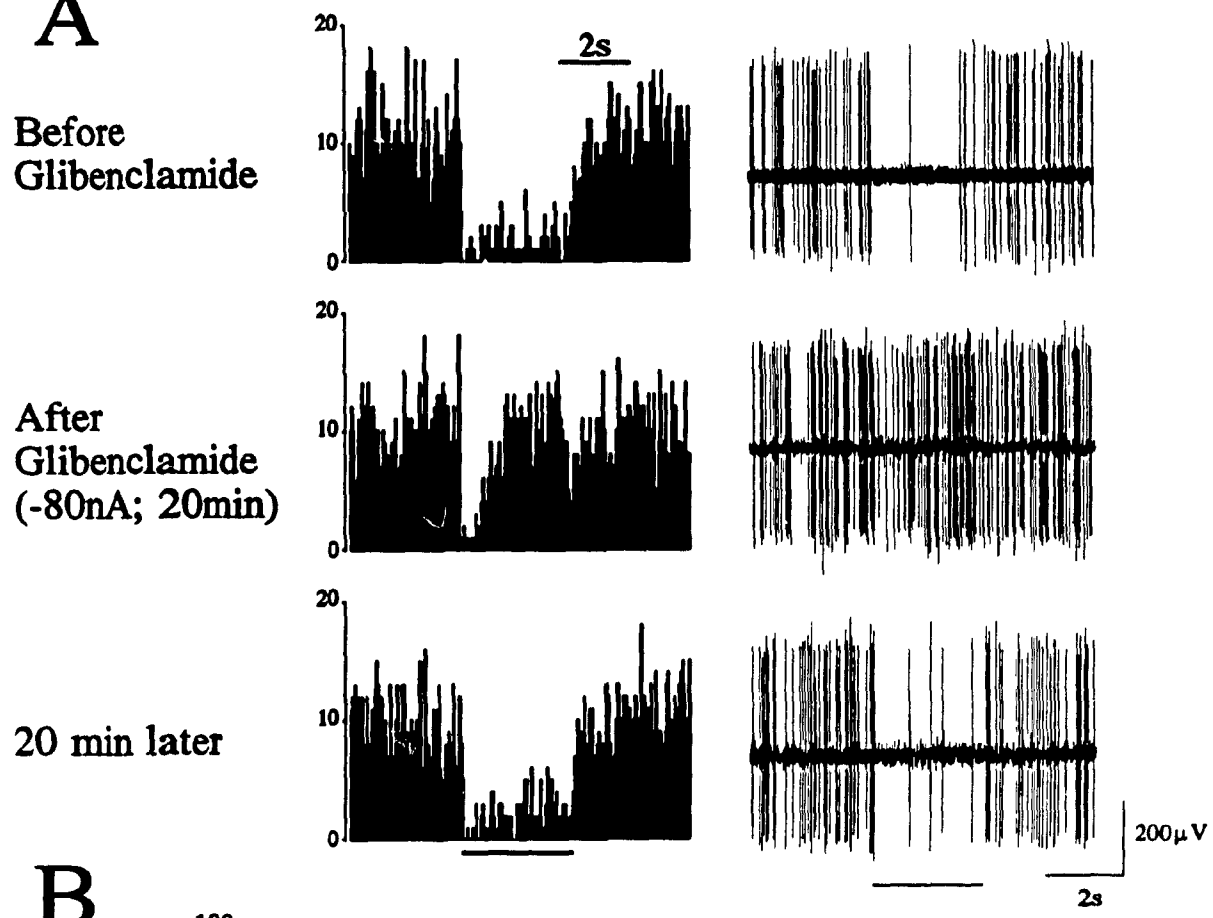
inhibition of action potential discharge caused by vibration was also greatly attenuated (Fig. 3A). By 30 min after the ATP injection, the IPSP had recovered to the control level as had the inhibition of action potential discharge. During the period when the IPSP was blocked the resting membrane potential, the amplitude of the action potential and the size of spontaneously occurring EPSPs (Fig. 3C) were unaffected, suggesting that the blockade of the IPSP was not a non-specific effect of ATP injection. The reversible blockade of the IPSP following ATP injection was reproducible with subsequent ATP injections (Fig. 3A). With two other neurones it was possible to maintain good recording conditions following ATP injection and in both cases the IPSP evoked by vibration was decreased after the injection. When recordings were made with electrodes containing only $K(CH_3SO_4)$ or KCl, no change in the IPSP evoked by vibration was seen after the end of hyperpolarizing current injection and the IPSP could be evoked reproducibly for up to 5 hours (De Koninck and Henry, 1988; chapter II).

Taken together the present results indicate that the IPSP evoked by vibration-sensitive afferent input is mediated postsynaptically by activation of ATP-sensitive K^+ channels. We have thus identified a physiological function for these channels in the central nervous system. Although, these channels have been described in other regions of the CNS (Miller, 1990) and it has been suggested that they may participate in the response to hypoxia (Amoroso *et al.*, 1990; Mourre *et al.*, 1990; Mourre *et al.*, 1989;

Ben-Ari *et al.*, 1990; Krnjević, 1990), a role for ATP-sensitive K^+ channels in a synaptic response had not been previously known.

Inhibition of nociceptive neurones in the dorsal horn by vibration provided a basis for the proposal that transmission in pain pathways may be controlled by input from large diameter primary afferents (Melzack and Wall, 1965). Vibration has subsequently been used widely to produce relief of clinical pain (Bini *et al.*, 1984; Lundeberg, 1984; Lundeberg *et al.*, 1984; Ottoson *et al.*, 1981). As the inhibition of spinal nociceptive neurones may be the physiological substrate for analgesic actions of vibratory stimulation (Salter and Henry, 1990a, 1990b; Wall and Cronly-Dillon, 1960a), a potentially important clinical implication of our results is that a cellular mechanism underlying this analgesia may be the activation of ATP-sensitive K^+ channels. Because there are newly-described pharmacological agents which act on these channels (Edwards and Weston, 1990), the present results may lead to novel pharmacological approaches to the treatment of pain.

Fig. 1 Iontophoretic application of glibenclamide attenuates the inhibition of ongoing activity evoked by cutaneously applied vibratory stimulation. **A:** Peristimulus time histograms (PSTHs) compiled before, just after and 20 min after the end of application of glibenclamide are shown on the left. Each PSTH was compiled using 15 consecutive stimuli. The width of each bin is 50 ms in all figures and the cumulative number of spikes per bin is shown on the ordinate. The period of application of the vibratory stimuli is indicated by the lines below the PSTHs. On the right are extracellular recordings showing representative single responses to vibration. These extracellular traces were sampled by computer continuously at 10 kHz. Note that the attenuation of the inhibition occurred with no change in the ongoing level of activity. **B:** Graph illustrating that repeated iontophoretic applications of glibenclamide each reversibly attenuates the inhibition by vibration. Each point is averaged for 15 consecutive inhibitory responses and shows the percentage inhibition, calculated from the rate of discharge during the 3 s period of the vibratory stimuli and the rate during a control period which was the 3 s period immediately prior to each vibration application. The periods of glibenclamide application are indicated by the lines above the points. For the first two applications glibenclamide was ejected by passing 50 nA of current and for the last application the ejection current was 80 nA. At the time indicated by the asterisk (*) the amplitude of the vibratory stimulus was increased from 350 to 500 μm. The data in B are taken from the same cell as in A.


A
Before Glibenclamide
After Glibenclamide (-80nA; 20min)
20 min later
2s
20
10
0
200μV
2s

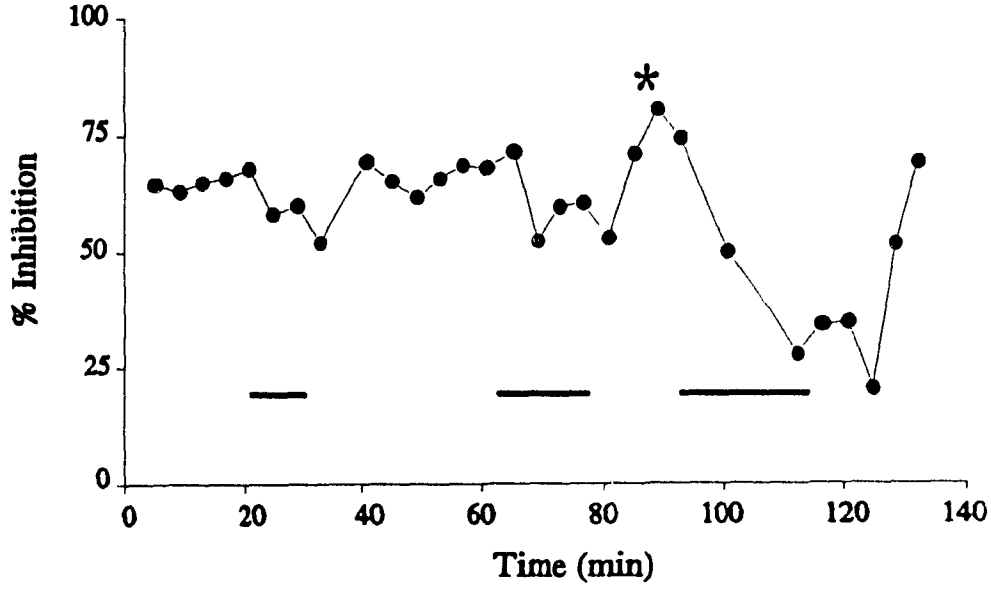
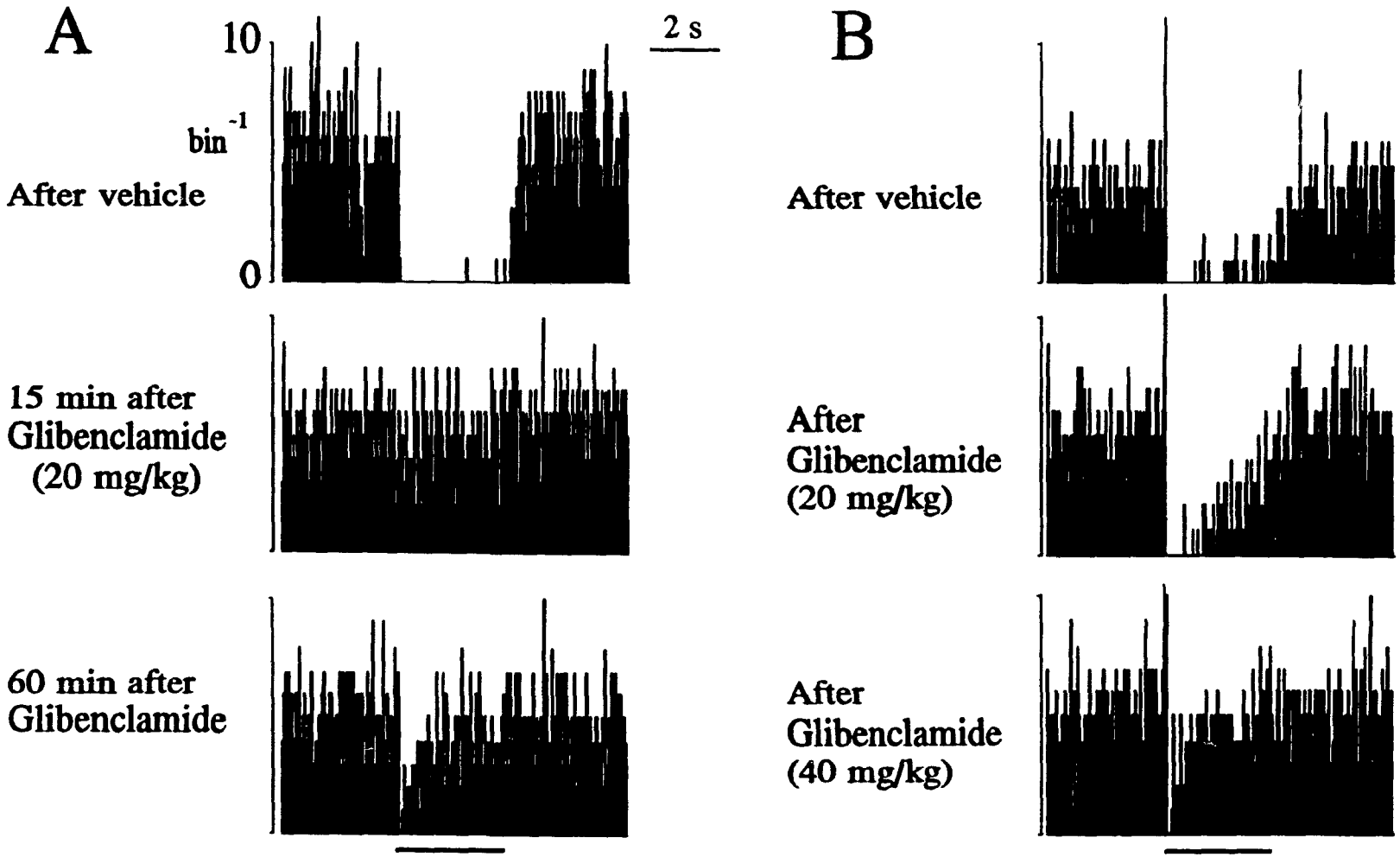

B
% Inhibition
100
75
50
25
0
0
20
40
60
80
100
120
140
Time (min)
*

Fig. 2 Intravenous administration of glibenclamide produces a reversible, dose-dependent blockade of the inhibition evoked by vibration. **A:** PSTHs illustrate the response to vibratory stimulation using an amplitude of 300 μm before, 15 min after and 60 min after the intravenous administration of glibenclamide (20 mg/kg). Note that the inhibition of firing was abolished 15 min after glibenclamide was administered and that partial recovery had occurred 45 min later. Complete recovery was observed in cases where the blockade by 20 mg/kg glibenclamide was only partial (not shown). **B:** With a different neurone, 15 min after administration of glibenclamide (20 mg/kg) the inhibition was reduced by 21%. This was the peak level of blockade in this case and therefore another dose of 20 mg/kg was then given. Following this dose the blockade increased to 76%. Each of the PSTHs was constructed using 10 periods of vibratory stimulation. The percentage blockade was calculated as (control inhibition - inhibition after glibenclamide)/ control inhibition. Glibenclamide, dissolved at 60 mg/ml in DMSO and diluted 1/10 in 150 mM NaCl and 50 mM NaOH, was administered over the course of 10-15 min. In all cases, the vehicle alone was administered prior to glibenclamide and in none did this control injection have any observable effect.


A
10
bin -1
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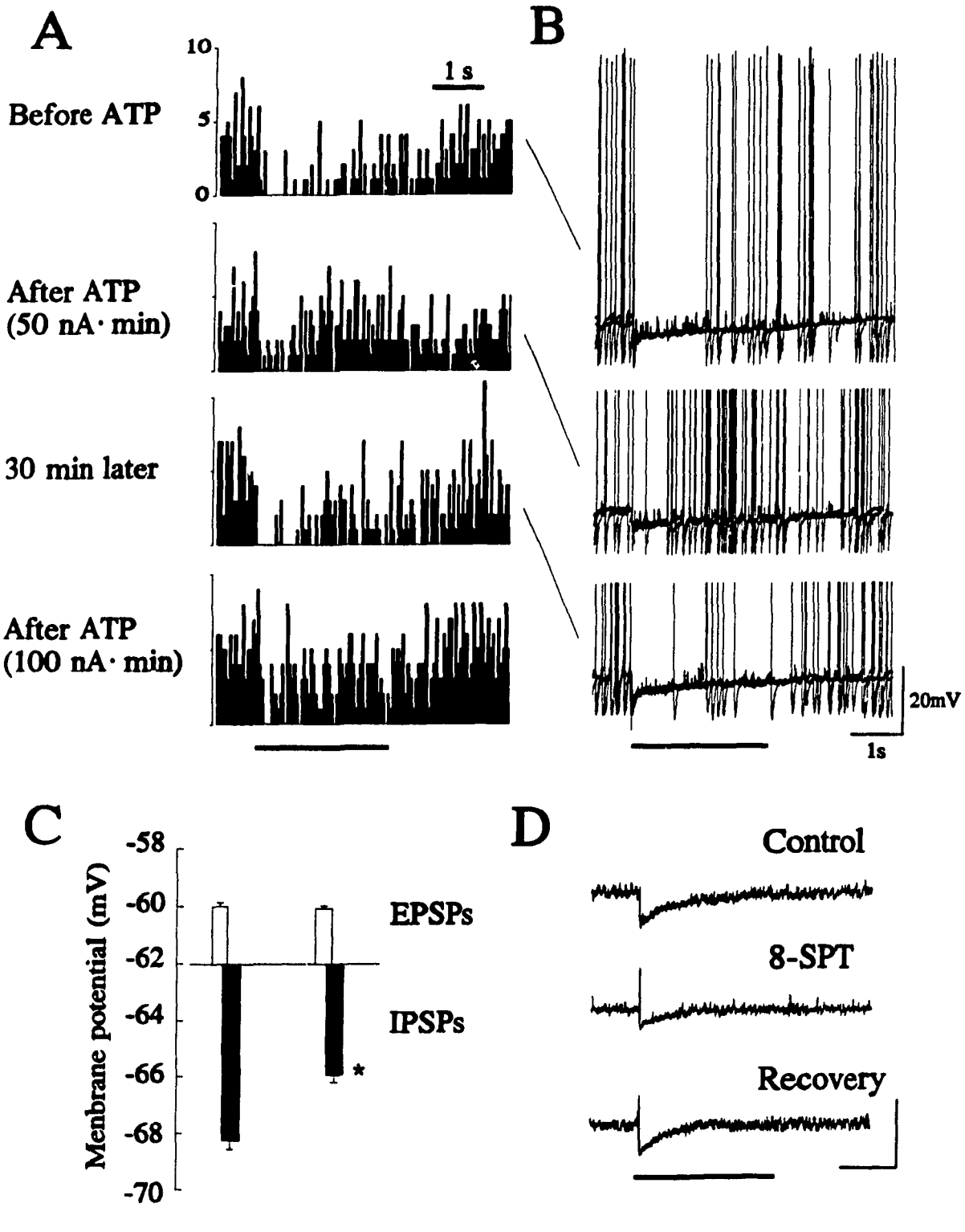
2 s
After vehicle
15 min after Glibenclamide (20 mg/kg)
60 min after Glibenclamide
B
After vehicle
After Glibenclamide (20 mg/kg)
After Glibenclamide (40 mg/kg)

Fig. 3 The IPSP evoked by vibration is blocked by intracellular injection of ATP and by extracellular application of 8-SPT. **A:** PSTHs illustrate that intracellular injection of ATP reversibly attenuates the inhibition of action potential discharge evoked by vibratory stimulation. The PSTHs were compiled using 15 vibratory stimuli before, 5 min after and 35 min after the injection of ATP (50 nA · min), respectively. Subsequently, a larger injection of ATP (100 nA · min) produced a greater attenuation of the inhibition (bottom PSTH). Thirty min after this injection the IPSP had recovered fully and was then blocked again by another 100 nA · min injection (not shown). The recording electrode contained 0.5 M ATP and 1.5 M $K(CH_3SO_4)$. The amplitude of the vibrational stimulus was 100 μm; the period of stimulation is indicated by the bar below the records. **B:** Each record is a set of 3 superimposed, consecutive intracellular traces illustrating that the vibration evokes an IPSP which is reversibly attenuated by the injection of ATP. The resting membrane potential was -62 mV and was unchanged after the injection of ATP. **C:** Histogram showing the average membrane potential at the peak of the IPSP (n=10, downward bars) evoked by vibration or at the peak of spontaneously occurring EPSPs (n=30, upward bars). The bars are mean $\pm$ SEM. Three EPSPs, the average number in a 1 s period, were measured just prior to each of the 10 stimuli used to determine the IPSP amplitude. Average IPSPs and EPSPs were measured before (left) and 5 min after (right) the injection of ATP (50 nA · min). Following injection of ATP the resting membrane potential remained at -62 mV and the IPSPs were significantly attenuated

($t=5.7$; $p<0.001$) whereas there was no significant change in the amplitude of the spontaneous the EPSPs ($t=0.43$; $p>0.5$). **D:** With another neurone, the IPSP evoked by vibration was attenuated by extracellular iontophoretic application of 8-SPT, as we have shown previously (De Koninck and Henry, 1988; chapter II). Each trace shows an average of 10 IPSPs before, immediately after and 15 min after extracellular application of 8-SPT. The peak of the IPSP was blocked by 48% by 8-SPT and there was no change in the resting membrane potential. 8-SPT was applied by iontophoresis (80 nA for 5 min) from a 3-barrelled micropipette which had been glued to the recording electrode such that the tips were separated by approximately 50 μm. Control application through a barrel containing 20 mM Tris-HCl had no effect on the magnitude of the IPSPs. In this case the recording electrode contained 3 M KCl. Action potentials have been filtered out for clarity of the illustration. The voltage and time scale in D is the same as in B.


A
B
10
5
0
1 s
Before ATP
After ATP
(50 nA· min)
30 min later
After ATP
(100 nA· min)
20mV
1s
C
-58
-60
-62
-64
-66
-68
-70
Membrane potential (mV)
EPSPs
IPSPs
*
D
Control
8-SPT
Recovery

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Wall, P.D. and Cronly-Dillon, J.R. (1960a) Pain, itch and vibration. *Arch.Neurol.* **2**:365-375.

CHAPTER IV

TWO PHYSIOLOGICALLY AND PHARMACOLOGICALLY DISTINCT IPSPs IN CAT DORSAL HORN NEURONES ELICITED BY LOW-THRESHOLD MECHANICAL STIMULATION: EVIDENCE FOR NATURALLY-ELICITED PURINE- AND GABA-MEDIATED IPSPs

ABSTRACT

$GABA_A$-mediated inhibition in dorsal horn neurones in response to low-threshold inputs have been reported but have not been fully characterized. In addition, an adenosine-mediated inhibition of dorsal horn neurones in response to low-threshold peripheral vibration has also been reported. Hence, as a low-threshold mechanical stimulus is likely to produce both types of inhibition we recorded extra- and intracellularly from dorsal horn neurones to characterize the post-synaptic inhibitions elicited by low-threshold mechanical pulses given outside the excitatory receptive field of the dorsal horn neurones. Inhibition of the on-going firing activity or IPSPs could be evoked from different locations outside the excitatory receptive field of each neurone. These IPSPs were characterized by 3 components, an early fast rising phase, a fast decaying phase (10-50 ms) and a slowly decaying phase (50-400 ms). Administration of bicuculline revealed that the IPSP was composed of an early bicuculline-insensitive component and a late bicuculline-sensitive component. Administration of caffeine blocked the bicuculline-insensitive component of the inhibition. Strychnine or naloxone had no effect on the inhibitions observed with the type of stimulus used in the present experiments. These results suggest the existence of adenosine- and $GABA_A$-mediated IPSPs in dorsal horn neurones in response to activation of low-threshold mechanical afferents. In addition, as pairs of mechanical stimuli given at varying intervals revealed a stronger depression of the $GABA_A$- than the adenosine- mediated component of the inhibition at interstimulus intervals of 1 s or less, it is suggested that the adenosine- and the GABA-mediated inhibitions are subject to different central regulatory mechanisms and that this may reflect their differential involvement in integration of sensory information.

INTRODUCTION

Different types of inhibition have been described in dorsal horn neurones following activation of low-threshold afferents. Hong *et al.* described IPSPs in unidentified neurones (1966) and in spinocervical tract neurones (1968) from low intensity electrical stimulation of several afferent nerves. Some IPSPs were shown to be Cl^- sensitive suggesting that they may have been mediated by $GABA_A$ or glycine mechanisms. Hong *et al.* (1968) also described Cl^- sensitive IPSPs from hair stimulation. Duggan and Foong (1985) described a bicuculline-sensitive inhibition of evoked responses in the dorsal horn following stimulation of the dorsal column. Game and Lodge (1975) observed bicuculline- and strychnine-sensitive inhibition of dorsal horn neurones following electrical stimulation of afferent nerves at intensities which recruit large myelinated afferents. Brown *et al.* (1987a) reported IPSPs from hair stimulation within and outside the excitatory receptive field of spinocervical tract neurones and also from activation of a single down-hair afferent outside the excitatory receptive field. They did not attempt to determine the membrane or chemical mechanisms involved in these types of IPSPs. These authors have also studied (1987b) the effects of paired inputs from single hair follicle afferents from within the excitatory receptive field of a single spinocervical tract neurone. They described a long lasting depression (> 1500 ms) of the excitatory response of dorsal horn neurones to the second of paired impulses in the primary afferent. This long lasting depression was attributed to a mechanism presynaptic to the spinocervical tract neurone (Brown *et al.*, 1987a). We have recently reported that this depression is

attenuated by intravenous administration of bicuculline, suggesting the involvement of a $GABA_A$ mediated inhibition (De Koninck and Henry, 1990).

We have also recently described an adenosine-mediated IPSP in dorsal horn neurones in response to low-threshold mechanical stimulation (vibration) applied in the periphery of the excitatory receptive field (Salter and Henry, 1985, 1987a, 1990a, 1990b; De Koninck and Henry, 1988).

The results of these studies suggest at least two types of pharmacologically distinct IPSPs in dorsal horn neurones in response to low-threshold mechanical stimulation. The present study was hence undertaken to further characterize these IPSPs in terms of their physiological, neurochemical and membrane biophysical properties. We recorded intra- and extracellularly from dorsal horn neurones *in vivo* and studied the inhibitory responses of these neurones to single and pairs of brief, low-threshold mechanical stimuli to the region of the skin outside the excitatory receptive field.

Preliminary results of this study have been presented elsewhere (Salter and Henry, 1988a; De Koninck and Henry, 1990)

METHODS

Animal preparation

Experiments were done on 33 cats anaesthetized with α-chloralose (60 mg/kg I.V.), sodium pentobarbitone (40 mg/kg, I.P.) or unanaesthetized, decerebrated after induction with halothane (Fluothane, Ayerst). The left common carotid artery and the left jugular vein were cannulated and a tracheal cannula was inserted. Spinal segments L_5-L_7 were exposed for recording and the spinal cords were transected at the L_1 vertebral level to eliminate the influence of supraspinal structures. Prior to the spinal transection, to minimize spinal shock the L_1 segment was injected with lidocaine hydrochloride (Xylocaine 1%, Astra; 0.1 mL). The exposed spinal cord was covered with a pool of warm mineral oil to prevent cooling and drying.

Arterial pressure was monitored continuously throughout the experiment and, when necessary, was maintained above 80 mmHg by intravenous infusion of dextran (Macrodex, Pharmacia; 6% in saline) or noradrenaline bitartrate (Levophed, Sterling-Winthrop Inc.; 0.002% in saline).

After bilateral pneumothorax, the animals were paralysed with pancuronium bromide (Pavulon, Organon; 1 mg/kg I.V.; repeated when required) and ventilated artificially. The level of anaesthesia was checked throughout the experiment by examination of the arterial pressure, the degree of pupillary constriction and by allowing the effects of the muscle relaxant to wear off periodically. When α-chloralose was used, an additional dose (30 mg/kg) was given I.V. 7-9 hours after the first injection and beyond

that time when required. When sodium pentobarbitone was used, additional doses (5 mg/kg, I.V.) were given every 3 hours. End tidal CO_2 concentration was monitored continuously using a Beckman LB2 Medical Gas Analyzer and was maintained between 3.5 and 5.0%. Rectal temperature was maintained at 38°C using a thermistor-controlled servo-mechanism and an infrared bulb.

Recording

Single glass micropipettes were used for the intracellular recording (3 M KCl or 2 M $K(CH_3SO_4)$; resistances 20-50 MΩ). Multibarrelled micropipettes were used for extracellular recording and iontophoresis of glutamate to raise the rate of firing of the neurones when required to enable observation of inhibition. The micropipettes were mounted on a stepping motor drive (Nanostepper). The electrodes were connected via locally build preamplifiers (DC preamplifier for intracellular recording, PCA-3; AC preamplifier for extracellular recording, HA-2; electronic workshop, Department of Physiology, McGill University) to a Tektronix 5111 oscilloscope. The amplified raw signal was recorded on magnetic tape using an Instrutech digitizer (VR-100) and a VCR tape-recorder. The output of the amplifier was also connected to a Data Translation DT2801-A analogue-to-digital board in a personal computer for sampling and analysis of selected evoked post-synaptic responses from peripheral natural and electrical stimulation. The software for data sampling and analysis were developed by MWS and YDK.

In some experiments single glass micropipettes filled with 3 M NaCl (resistance: 1-3 MΩ) were used to record the cord dorsum potential at the site of entry of the dorsal roots.

Criteria for good intracellular recording were as follows: penetration with a shift in DC potential of at least 40-50 mV upon penetration, a recovery from injury spike within a few seconds, a stable resting membrane potential of 50-65 mV and spikes with overshoot.

Intravenous administration of antagonists

Antagonists were administered intravenously via the catheter in the external jugular vein. Intravenous administration was preferred to iontophoretic administration to ensure that the antagonist would reach a wide number of its receptor sites. The dosages were as follows: bicuculline hydrochloride (Research Biochemicals Inc.), 0.3-1.0 mg/kg (De Koninck and Henry, 1990; Duggan and Foong, 1985; Salter and Henry, 1988a); strychnine sulphate (British Drug Houses), 0.2-0.6 mg/kg; naloxone hydrochloride (Narcan, Endo), 0.1-0.5 mg/kg (Calvillo *et al.*, 1974; Calvillo *et al.*, 1979); caffeine (Fisher), 20-60 mg/kg (Salter and Henry, 1987a). In experiments where antagonists were administered intravenously, the cats were pretreated with hexamethonium bromide (10 mg/kg I.V. every 5 hours; Sigma) to block the changes in blood pressure induced by bicuculline (Hong and Henry, 1990) and strychnine (Hong *et al.*, 1989).

Classification of neurones

Each unit was carefully classified according to its responses to natural and electrical peripheral stimulation. In some experiments, the peroneal, tibial and sural nerves were exposed at the level of the ankle ipsilateral to the side of recording for electrical stimulation. The fur on the foot was unevenly cut to leave some hairs but also to expose the skin in places for natural stimulation.

The natural stimuli used were: movement of hair, innocuous touch/pressure, noxious pressure of the foot or the paws, noxious pinch of the skin with a serrated forceps and noxious radiant heat. According to its responsiveness, each unit was classified as non-nociceptive, wide dynamic range, nociceptive specific or proprioceptive. Non-nociceptive neurones responded only to innocuous stimuli, wide dynamic range neurons responded to both innocuous and noxious stimuli and nociceptive specific neurons responded only to noxious stimuli. Both wide dynamic range and nociceptive specific neurons were considered to be nociceptive.

Low-threshold mechanical stimulation outside the excitatory receptive field

A feedback-controlled mechanical stimulator (Chubbuck, 1966) was apposed on the skin outside the excitatory receptive field and mechanical displacements of 10-1000 μm (5 ms duration) were used for low-threshold mechanical stimulation.

RESULTS

Recording from 45 neurones are included in these results; 26 neurones were recorded extracellularly and 19 intracellularly. No difference was observed with respect to the responses studied in neurones taken from animals anaesthetized with chloralose or pentobarbitone or from unanaesthetized (decerebrate) animals and hence the data from all three preparations are considered together.

Inhibition evoked by low-threshold mechanical stimulation outside the excitatory receptive field

IPSPs or inhibition of the firing activity could be observed with 35 dorsal horn neurones in response to low-threshold mechanical stimulation when the probe was placed outside the excitatory receptive field of the neurone. Fig. 1 gives an example of IPSPs which could be obtained from mechanical stimuli at different locations outside the excitatory receptive field of the dorsal horn neurone. These IPSPs were usually characterized by an initial fast rising and fast decaying phase (10-50 ms) and a later slowly decaying phase (50-400 ms). Note also, in the case of the neurone in Fig. 1, that brief touch movements in the excitatory receptive field which evoked EPSPs (sometime giving rise to an action potential) could also evoke IPSPs. Several complex responses could be observed consisting of mixed excitation and inhibition when stimulating inside the excitatory receptive field. Stimulation outside the excitatory receptive field produced either pure inhibition or inhibition followed by some excitation. As the focus of the

present study is on the characterization of the IPSPs, for each neurone studied, a location outside the excitatory receptive field was chosen so that IPSPs could be obtained with the least amount of excitation.

Effect of bicuculline on inhibition evoked by mechanical stimulation

In the 25 cells tested (16 extracellularly and 9 intracellularly), administration of bicuculline (0.3-1.0 mg/kg, *i.v.*) revealed, in 80% of the cases, that the inhibition obtained in response to low-threshold mechanical stimulation could be dissected into two components: an early short (10-25 ms) bicuculline-insensitive component and a later, longer (50-400 ms) bicuculline-sensitive component (Fig. 2). The early component had a latency to peak of 12-20 ms after the onset of the mechanical stimulus. This component decayed rapidly to last 10-50 ms. The later component had a latency to peak of 25-50 ms and it decayed slowly to last 20-400 ms. In the remaining 20% of the cells tested, an early bicuculline-insensitive inhibition was not detectable. In cases were intracellular electrodes filled with KCl were used, the later component of the IPSP rapidly reversed from hyperpolarizing to depolarizing within 5-10 min of the recording confirming that it was mediated by a Cl^- conductance consistent with its mediation by a $GABA_A$ mechanism.

IPSPs evoked by paired mechanical stimuli

To characterize further the physiological properties of these IPSPs, we used pairs of mechanical stimuli given at different interstimulus intervals (12 cells extracellularly; 8 intracellularly). The IPSP in response to the second of the pair of stimuli was depressed most at shorter intervals. Depression of the second IPSP was seen at intervals of up to or more than 1 s. The duration of the depression clearly outlasted the duration of the IPSP itself.

After administration of bicuculline (0.3-1.0 mg/kg), the remaining IPSP was not as depressed at short interstimulus intervals (Fig. 3B, 4), suggesting that the frequency response of the system leading to the bicuculline-insensitive IPSP was greater than that of the system leading to the bicuculline-sensitive IPSP.

In some cases, when applying the mechanical stimulus outside the primary excitatory receptive field the inhibitory response was often contaminated with an excitatory response. Fig. 4 illustrates this phenomenon. One can observe, with intracellular recording, first an early brief IPSP followed by an EPSP. Note that, at shorter interstimulus intervals, the EPSP is dramatically depressed allowing the early component of the IPSP to broaden (its recovery phase had been masked by the EPSP). Hence the early component of the IPSP can clearly be differentiated from the EPSP in that this early IPSP is not attenuated at short interstimulus intervals.

Comparison of the latency of the IPSP with that of the EPSP

The fact that the early component of the IPSP occurs before the EPSP could, at first sight, suggest that this component of the IPSP is monosynaptic, which is contrary to current belief. However, the EPSP observed in the present situation results either from activation of subliminal input around the primary receptive field or from a spread of the mechanical stimulus to the excitatory receptive field. With this type of approach, it is impossible to evaluate the central delay for each component of the response to mechanical stimulation by recording an afferent field, as it probably represents a mixed afferent field. For comparison, we also located the mechanical probe on the hairs in the primary excitatory receptive field in an attempt to observe the shortest latency EPSP possible. An example of the records obtained is illustrated in Fig. 4B. The frequency of stimulation was increased to reduce the EPSP to its early components. Note that the onset of the IPSP occurs at a comparable or slightly longer latency than the earliest EPSP. The peak of the IPSP however occurs before the onset of the second EPSP (see inflection point in the rising phase of the EPSP complex).

Reversal potentials of the early and late IPSPs

The early component of the IPSPs described above remains after bicuculline administration, suggesting that it does not involve a $GABA_A$-mediated mechanism. In addition, its extrapolated reversal potential (Fig. 5B) was clearly below the reversal

potential of the late component (Fig. 5A), suggesting that the early component did not involve a Cl^- conductance. The reversal potential of this early component is consistent with an increase K^+ conductance. Our previously reported vibration-induced, adenosine-mediated IPSP was also bicuculline-resistant and had a reversal potential suggesting the participation of a K^+ conductance (De Koninck and Henry, 1988; chapter II; Salter and Henry, 1987a). Hence we tested caffeine, a P_1-purinergic receptor antagonist, on the early bicuculline-insensitive IPSP.

Effect of caffeine on the early component of the inhibition

As opposed to bicuculline, the effects of caffeine on the blood pressure are not blocked by hexamethonium. Accordingly, it was impossible to maintain intracellular recording after intravenous injection of caffeine. However, an inhibition could be observed extracellularly which was analoguous to the IPSP observed intracellularly (cf. Figs. 5 and 6). Glutamate was applied by iontophoresis to raise the on-going firing activity of the neurones to allow better visualization of the inhibition. As the inhibition was thus observed on glutamate-evoked activity, this suggests that the inhibition is postsynaptic and therefore reflects the IPSPs observed intracellularly. Because of this and because the inhibitions recorded intracellularly and extracellularly showed the same dynamic properties (see Fig. 7), the data obtained from these two recording methods are considered together. Therefore, the effects of caffeine were tested on the inhibition of

the rate of on-going activity recorded extracellularly. Caffeine reversibly blocked the early bicuculline-insensitive inhibition in all the 4 cells tested, suggesting that it involved an adenosine-mediated mechanism (Fig. 6). Administration of strychnine (n=4) or naloxone (n=5) had no effect on either component of the inhibition.

Frequency dependence of the early and late components of the inhibition

The similarity between the results obtained from the extra- and intracellular recordings suggests that the properties observed intracellularly were not due to artifacts of the intracellular recording. As previously suggested by the results from paired pulse stimulation, the early adenosine-mediated component of the inhibition appears to follow higher frequencies of stimulation than the later component. This is illustrated in Fig. 7, where peristimulus time histograms were made of the response to mechanical stimulation at different frequencies. Note at low intensity of mechanical stimulation (Fig. 7A) the differentiation between the early and the late components of the inhibition. Note also that the early component persists at higher frequency (see also Fig. 8A). At a higher intensity mechanical stimulation (Fig. 7B), the inhibition resembled the IPSP shown in Figs. 1-3. It is the later component of the inhibition that is the most depressed at a higher frequency of stimulation (see also Fig. 8B).

DISCUSSION

In the present study, we have demonstrated two IPSPs in dorsal horn neurones in response to activation of low-threshold mechanosensitive afferents, an early, short bicuculline-insensitive IPSP which appears to be mediated by an increase in a K^+ conductance and a later, longer bicuculline-sensitive IPSP mediated by a Cl^- conductance. Game and Lodge (1975) reported bicuculline- and also strychnine-sensitive inhibitions of deep dorsal horn neurones in response to low-threshold electrical stimulation of afferents nerves. With the stimuli used in the present experiment, we did not observe such inhibitions or IPSPs attenuated by strychnine. This may suggest that the electrical stimuli used by Game and Lodge activated another class of afferents, which specifically gave rise to a glycine mediated IPSP and that this class of afferent was not activated with the mechanical stimuli used in our study.

Differentiation of purinergic and GABAergic IPSPs

The early K^+-mediated IPSP has characteristics similar to those of the IPSP that we have previously described in response to peripheral vibration stimulation (Salter and Henry, 1987a; De Koninck and Henry, 1988; chapter II). This IPSP is also similar to the responses to adenosine that we have observed in the dorsal horn *in vivo* (Salter and Henry, 1985; De Koninck and Henry, 1988; chapter II) and that have been reported in other systems (Segal, 1982; Lotan *et al.*, 1982; Greene and Haas, 1985; Gerber *et al.*,

1989; Spuler and Grafe, 1989). As this early inhibition is specifically blocked by caffeine, a P_1-purinergic receptor antagonist, as is the inhibition induced by vibration (Salter and Henry, 1987a; chapter II) and by adenosine (Salter and Henry, 1985), we therefore suggest that this early, K^+-mediated IPSP is mediated by endogenous adenosine. Accordingly, we shall refer to the early component of the inhibition to low-threshold mechanical stimulation as the *purinergic* component. The later, slower component of the inhibition which does not follow higher frequencies will be referred to as the GABAergic component because it is blocked by bicuculline and is mediated by a Cl^- conductance.

Time course of the IPSPs

The purinergic IPSP reported here was markedly shorter than the GABAergic component. The duration of the IPSPs reported here was often longer than previously reported IPSPs in dorsal horn neurones from electrical stimulation of afferent nerves (Hongo *et al.*, 1966, 1968) and natural stimulation (Brown *et al.*, 1987a) using air jets. However, the longer IPSPs reported here correspond to stimulation at intensities greater than those (air jets) used by Brown *et al.* (1987a). The latter authors also did not attempt to stimulate in locations were pure IPSPs were not contaminated by EPSPs. In addition, Brown *et al.* (1987a) did not average their IPSPs which renders it difficult to visualize precisely the tail end of the IPSP, especially in view of the occurrence of large spontaneous EPSPs recorded in dorsal horn neurones (see Figs. 2 and 3 for example).

The IPSPs from electrical stimulation of afferent nerves reported by Hongo *et al.* (1966, 1968) lasted usually up to 60-80 ms which is again shorter than the longer ones reported here. However, it must be mentioned that they often used conditions where the membrane potential of the neurone had deteriorated to a depolarized level where the action potential generating mechanism was blocked to avoid contamination of the responses with the action potential. In such recording conditions, presumed greater damage in the membrane would cause a greater shunt in input resistance which may mask again the tail end of their IPSPs.

The purinergic component of the IPSPs reported here was markedly shorter than the GABAergic component. It is interesting to note that, of all the IPSPs recorded by Hongo *et al.* (1966, 1968) that they have recorded, those IPSPs evoked from stimulation of the interosseous nerve (which is mainly constituted by fibres innervating Pacinian corpuscles) with characteristically shorter duration than the IPSPs evoked from stimulation of other afferent nerves. This common feature between the purinergic IPSP reported here and the IPSPs in response to interosseous nerve stimulation may suggest a link between this type of IPSP and Pacinian afferents (see discussion below).

Frequency response relationship

GABAergic IPSP

We observed a strong depression of the GABAergic IPSP when pairs of mechanical stimuli are given at interstimulus intervals up to 1 s or more. Thus, this depression vastly outlasts the duration of the IPSP itself, suggesting that it was not due to the IPSP itself, but either to a mechanism presynaptic to the neurone recorded from or due to a postsynaptic mechanism other than one involving a change in membrane potential or resistance, such as receptor desensitization.

The same type of long lasting depression of the postsynaptic response was also seen with EPSPs to stimulation of low-threshold mechanical afferents (see also Tapper *et al.*, 1983; Brown *et al.*, 1987b; Noble and Short, 1989; Short *et al.*, 1990; De Koninck and Henry, 1990; chapter V). The latter depression was seen at interstimulus intervals greater than 1 s, intervals longer than the duration of the longest IPSP observed. In addition, prolonged depression of paired excitatory responses to impulses in single afferents was not associated with an apparent postsynaptic inhibitory potential (Tapper *et al.*, 1987; Brown *et al.*, 1987a; De Koninck and Henry, 1990; chapter V). However, such depression was attenuated by administration of bicuculline (De Koninck and Henry, 1990; chapter V), suggesting that it involves a $GABA_A$-mediated mechanism presynaptic to the cell recorded from. It is possible that the depression of the second of paired IPSPs observed in the present study is due to a similar mechanism. This would suggest that the

postsynaptic GABAergic response is in turn affected by the general GABAergic system triggered from activation of low-threshold afferent input. However, as the GABAergic IPSP studied here is sensitive to bicuculline, this possibility cannot be tested.

Purinergic IPSP

With respect to the early short purinergic component of the inhibition, the depression of the second of paired IPSPs or to repetitive stimulation was much weaker than that seen with the GABAergic component. This may suggest that the system (network) leading to this IPSP is not under the same inhibitory control as the GABAergic IPSP and therefore encodes for events with greater repetitive frequency. As mentioned earlier, the purinergic IPSP described here appears to rely on the same mechanisms as our previously described prolonged, vibration-induced purinergic IPSP (Salter and Henry, 1987a; De Koninck and Henry, 1988; chapter II). Thus, the observation that the short purinergic IPSP described in the present study is only weakly inhibited upon repetitive stimulation suggests that the prolonged vibration-induced IPSP may be composed of a train of these short purinergic IPSPs.

It is also interesting to note that, in the cases of EPSPs the depression of the central response to the second of paired impulses in single afferents is most prominent with guard-hair afferent input as opposed to input from down-hair or other mechanosensitive afferents (Tapper *et al.*, 1983; Brown *et al.*, 1987b; Koerber and

Mendell, 1988; De Koninck and Henry, 1990; chapter V). It has been suggested that this greater sensitivity of the central response to repetitive guard-hair input was due to the fact that this response involved the activation of a large polysynaptic component which is more sensitive to repetitive stimulation (Hongo and Koike, 1975; Brown *et al.*, 1987a; Koerber and Mendell, 1988; chapter V). Hence, as the duration of the purinergic IPSP is much shorter than that of the GABAergic IPSP, and by analogy with the studies on EPSPs mentioned above, it may be suggested that the mechanism giving rise to the purinergic IPSPs relies on a simpler central network than that giving rise to the GABAergic IPSPs. However, it remains possible that the difference in time course and frequency sensitivity of the two IPSPs relies on the properties of the chemical mechanisms underlying them.

Amplitude response relationship

The results illustrated in Fig. 7 and 8 suggest that the relative participation of the GABAergic and the purinergic components in the overall inhibition varies with the amplitude of the stimulus. The duration of the IPSP following low-threshold primary afferent stimulation increases in duration with the amplitude of the stimulus. The magnitude of the peak inhibition also increases. Interestingly, at higher amplitudes of stimulation, it is the late GABAergic component of the inhibition that is most affected by the increase in the frequency of stimulation. Hence, with greater intensity the early purinergic component appears to constitute a greater proportion of the overall inhibition

to mechanical stimulation, and at smaller intensity it is the GABAergic component of the inhibition that plays the major role. However, this comparison applies only to the type of stimulus used in the present experiment.

Neuronal substrate underlying each IPSP

Afferents involved

GABAergic IPSP. Hongo *et al.* (1966; 1968) reported Cl^--sensitive IPSPs from hair stimulation in an inhibitory region of the receptive field of spinocervical tract neurones. Brown *et al.* (Brown *et al.*, 1987a) did not observe any IPSP in response to activation of single guard-hair afferents within or outside the excitatory receptive field of spinocervical tract neurones, but do not exclude the possibility that such IPSPs may occur. Recently, we have seen one case of pure inhibition in response to activation of a single guard-hair afferent arising from an inhibitory region of the receptive field of the dorsal horn neurone (De Koninck & Henry, unpublished observation). This inhibition showed the same sensitivity as the GABAergic IPSP to pairs of impulses in the primary afferent at intervals of 500 ms. Brown *et al.* (1987a) also report one IPSP from a single down-hair afferent from outside the excitatory receptive field of the dorsal horn neurone. Finally, Tapper *et al.* (1987) did not observe any IPSP in dorsal horn neurones in response to activation of single type 1 slowly adapting mechanoreceptors. The latency of the GABAergic IPSP reported in the present study is too short to correspond solely to

down-hair (Aδ conduction velocity) activation. It seems probable therefore that the GABAergic inhibition observed in the present study may be caused by the activation of guard-hair afferents from an inhibitory region of the receptive field of the dorsal horn neurone. However, the participation of other types of afferent in triggering the occurrence of such an IPSP cannot be excluded.

Purinergic IPSP. In earlier parametric studies (Salter and Henry, 1990a), based on the preferential frequencies and amplitudes at which the vibration-induced purinergic inhibition was obtained, it was suggested that the inhibition may result from the activation of Pacinian corpuscule afferents. Hence, as the purinergic IPSP described in the present study may be the same as the inhibition we described earlier in response to vibration (Salter and Henry, 1987a, 1990a; De Koninck and Henry, 1988; chapter II) and if we consider the possibility that these purinergic IPSP may occur primarily in response to a single type of afferent, this would suggest that these IPSPs are linked to Pacinian afferent activation. Other studies have reported IPSPs in response to 500 Hz stimulation of the glabrous skin (Brown *et al.*, 1987a) and to electrical stimulation of the interosseous nerve (Hongo *et al.*, 1968), which is consistent with Pacinian afferent activation (Hunt and McIntyre, 1960; Hunt, 1961).

Neuronal circuitry

IPSPs in response to primary afferent activation are usually attributable to di- or polysynaptic mechanisms, involving at least one interneurone in the pathway. Hence, the

IPSPs considered here would possibly involve GABAergic or purinergic interneurones. GABAergic interneurones have been reported in the dorsal horn (McLaughlin *et al.*, 1975; Barber *et al.*, 1978; Ribeiro-da-Silva and Coimbra, 1980; Todd and McKenzie, 1989) and one report suggests the possibility of purinergic interneurones in the dorsal horn (Braas *et al.*, 1986). Recurrent inhibition seems unlikely to be the explanation for these IPSPs as they were observed in the absence of a preceding excitatory responses. In addition, Hongo *et al.* (1968) also did not observe IPSPs from stimulation of the thoracic cord at intensities just subthreshold for the antidromic invasion of the spinocervical tract neurones they recorded from.

In previous studies, we have suggested that the extracellular hydrolysis of ATP may represent the source of endogenous adenosine implicated in the mediation of the purinergic IPSP (Salter and Henry, 1985, 1990a). Such an hypothesis was based on the similarity of the effects of exogenously applied ATP and the response to vibration. ATP was only excitatory when applied in the vicinity of non-nociceptive neurones and had a biphasic effect (excitation followed by inhibition) or an inhibitory effect only when applied in the vicinity of nociceptive neurones (Salter and Henry, 1985). The same separation of effects was seen with vibration on nociceptive and non-nociceptive dorsal horn neurones (Salter and Henry, 1990b). Both the inhibitory effect of ATP and the inhibitory effect of vibration are blocked by P_1-purinergic antagonists (Salter and Henry, 1985,

1987a) and both are specifically potentiated by tachykinins (Salter and Henry, 1987b, 1988b).

It has also been suggested that ATP may be released from low-threshold primary afferents (Fyffe and Perl, 1984; Holton and Holton, 1954). ATP released from low-threshold primary afferents and hydrolysed to adenosine (Bruns, 1980; Salter and Henry, 1985) to act directly on dorsal horn neurones could therefore represent the neuronal substrate for the purinergic IPSP presented here.

Functional relevance

Whatever the pathway involved in the mediation of the two IPSPs described in the present study, it is interesting to note that each seems to rely on mechanisms with different dynamic properties. Hence, each may reflect central responses specific to different stimuli and as they may correspond to activation of different classes of afferents, it illustrates that the dynamic properties of the central response may be specific to the peripheral receptors activated.

Fig. 1. IPSPs from low threshold mechanical stimulation at different locations on the hind foot in and around the excitatory receptive field of a single dorsal horn neurone. The IPSPs illustrated were obtained from averages of 10 responses to a 100 μm mechanical stimulus applied to the skin at the locations indicated by the dark lines. The excitatory receptive field of the neurone is illustrate by the hatched area. The lower trace illustrates the response to sustained hair stimulation (long bar below the trace; constant displacement) and to brief pulses (short bars below the trace).

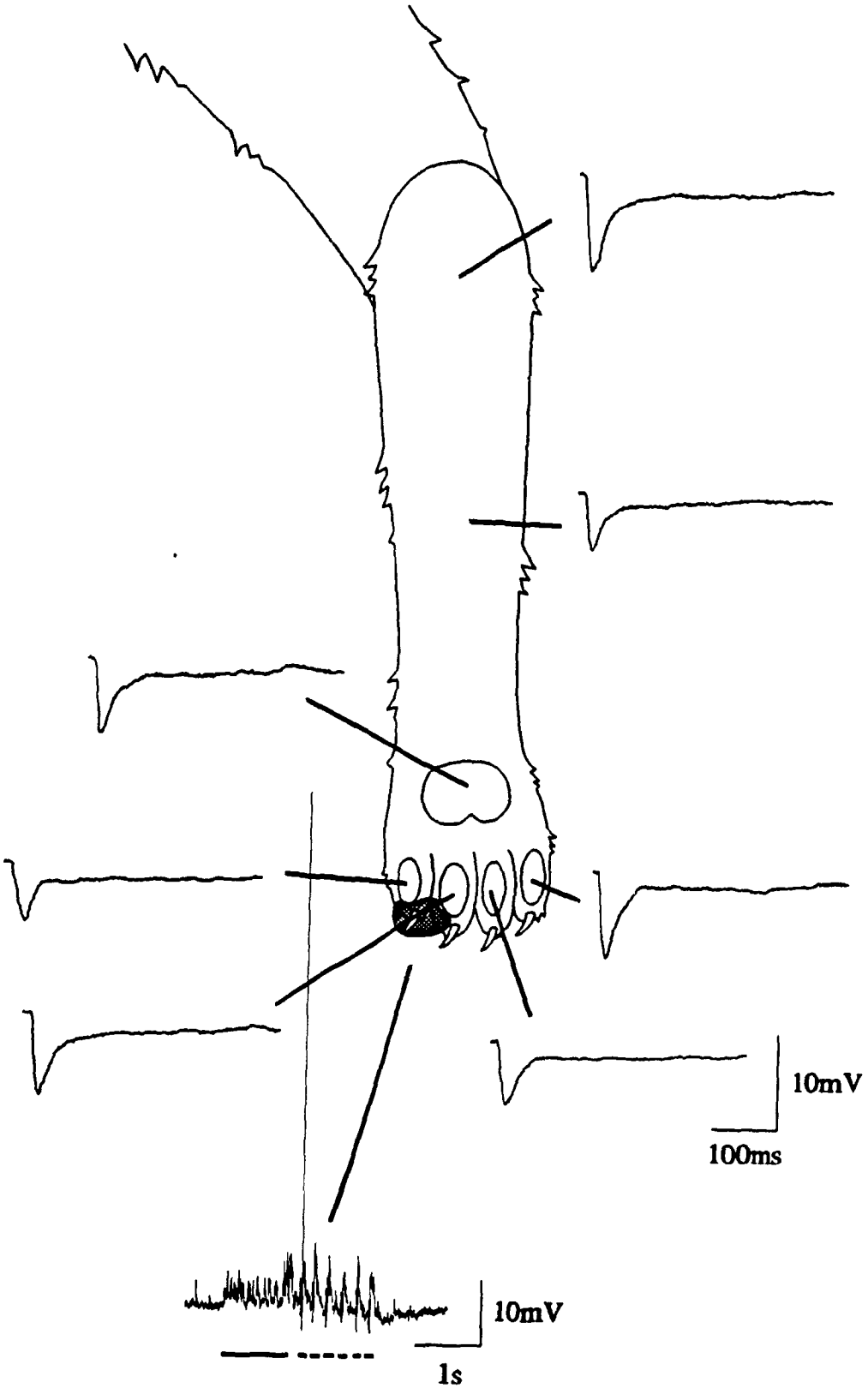

10mV
100ms
10mV
1s

Fig. 2. IPSP recorded in a dorsal horn neurone in response to a low-threshold mechanical stimulus applied outside the excitatory receptive field. Two traces are direct recording before and after bicuculline administration (1.0 mg/kg I.V). The third trace was calculated by subtraction of the trace after bicuculline from that before. Each trace represents 20 averaged IPSPs. The onset of the stimulus is at the beginning of each trace. The resting membrane potential of the neurone was -58 mV and did not change upon administration of bicuculline. Recording was with a $K(CH_3SO_4)$ filled electrode.

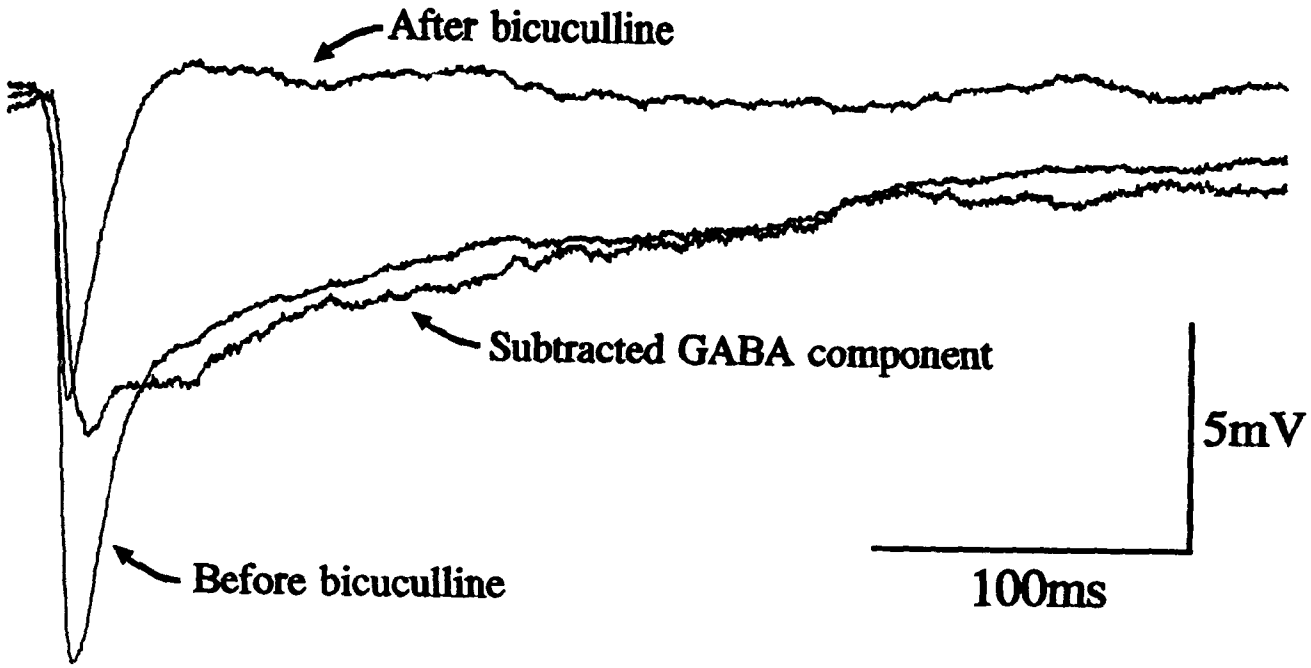
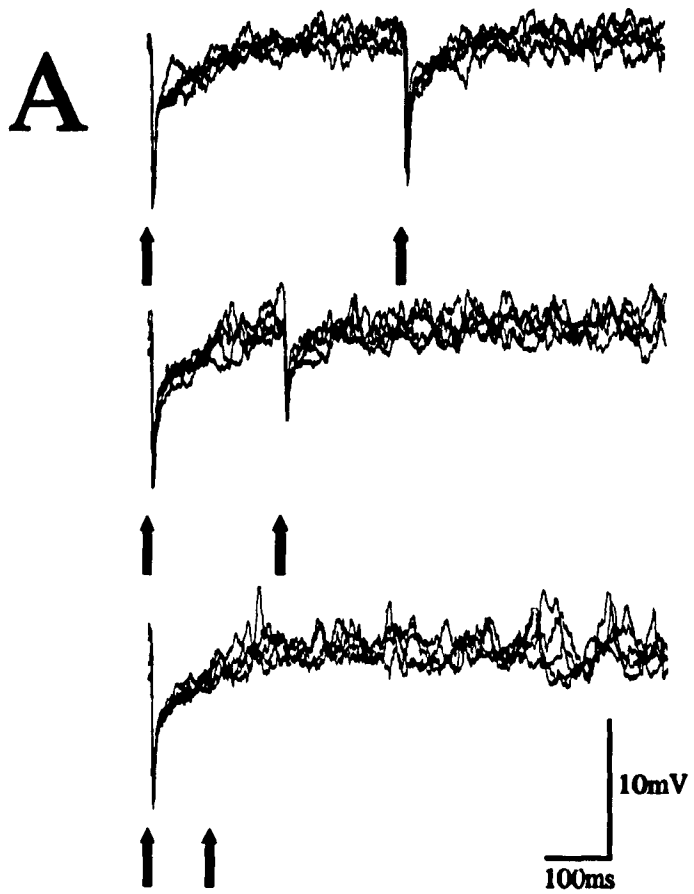

After bicuculline
Subtracted GABA component
5mV
Before bicuculline
100ms

Fig. 3. IPSPs in response to pairs of low-threshold mechanical stimuli given at different intervals. Each record shows superimposed, consecutive traces. Same neurone as in Fig. 1. The resting membrane potential of the neurone was -58 mV. In *A*, pairs of brief mechanical stimuli were given at interstimulus intervals of 750, 400 and 200 ms, respectively from top to bottom. Arrows point to the time of onset of each mechanical mechanical stimulus. Note that the depression of the second of paired IPSPs is greater at shorter interstimulus intervals, but still occurs even at 750 ms. In *B* the response at 400 ms was recorded before (*top trace*) and after (*bottom trace*) administration of bicuculline (1.0 mg/kg I.V.). The spiking activity increased slightly after bicuculline injection but no change in resting membrane potential was observed. Action potentials are truncated. Recording was with a $K(CH_3SO_4)$ filled electrode.


A
10mV
100ms

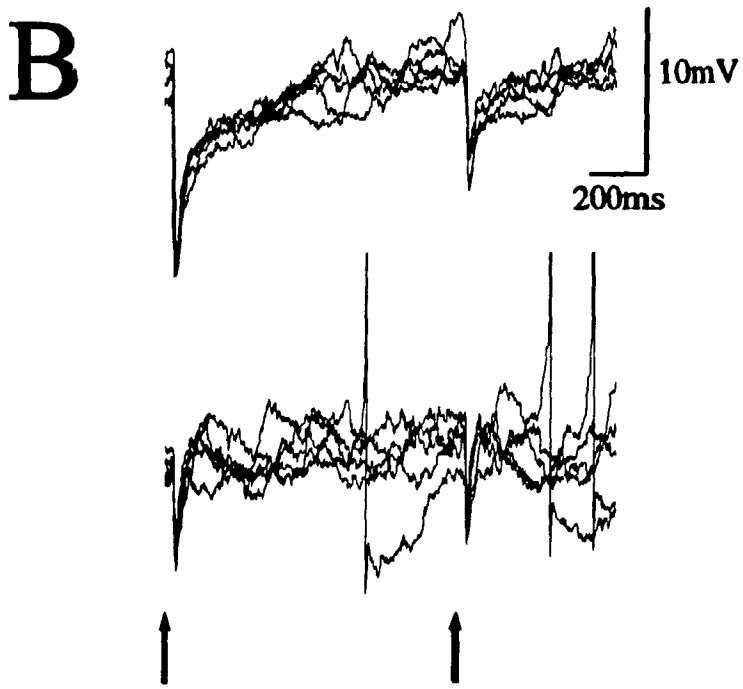

B
10mV
200ms

Fig. 4. Mixed response to pairs of low-threshold mechanical stimuli given at different intervals. Each trace in *A* represents four superimposed consecutive traces. In *A*, pairs of brief mechanical stimuli were given at intervals of 1 s, 500, 200 and 70 ms. Arrows point to the time of onset of displacement of the mechanical stimulator. The inset shows an enlargement of the area in the square box. Note the early IPSP followed by an EPSP. The EPSP is depressed at shorter interstimulus intervals; the early IPSP not only is not depressed, but its full recovery phase is unmasked. The mechanical probe was placed at the location marked on the diagram in *B* (*arrow a*). The diagram in *B* represents the receptive field of the neurone: the darkened area represents the low-threshold (hair) receptive field of the neurone and the greater hatched area the high-threshold (pinch) receptive field. The response from stimulating at location *a* was compared with the response to stimulation when the probe was placed on hair in the excitatory receptive field (*arrow b*) to observe the fastest EPSP to mechanical stimulation (*trace a*). Traces *a* and *b* show averages of 30 traces (stimulation at 10 Hz).

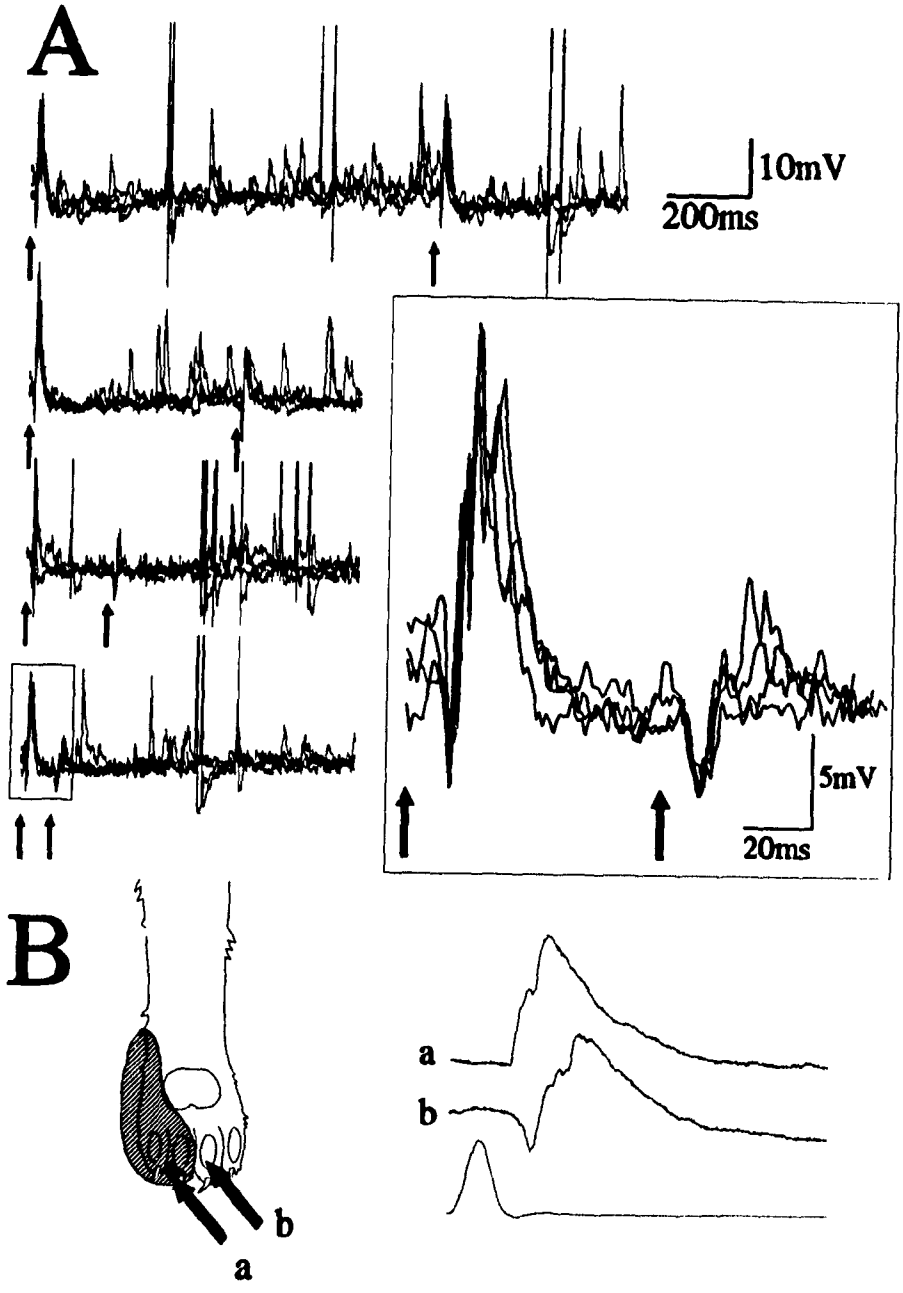

A
10mV
200ms
5mV
20ms
B
a
b
a
b

Fig. 5. Reversal potential of the different components of the IPSP; evidence for Cl^-- and K^+-mediated components. **A:** Reversal potential of the IPSP Recorded with a $K(CH_3SO_4)$ filled electrode. The traces on the left represent voltage deflections used to build the graph on the right. Each of the 4 traces is an average of 5 responses to mechanical stimulation. The open circles represent the membrane potential immediately before the onset of the IPSP and the closed circles, the potential at the peak of the IPSP. The late component of the IPSP reverses at a potential less than -65 mV, but note the persistent early component. Hence the peak of the IPSP at depolarised potentials is a sum of both components, whereas the inverted peak at hyperpolarized potentials is due to the Cl^- component. This latter observation may account for the apparent rectification in the size of the IPSP at depolarized potentials (graph). **B:** Reversal potential of the IPSP from another neurone recorded with a KCl filled electrode, with an emphasis on the early K^+-mediated component. Note the extrapolated reversal potential more negative than -90 mV.

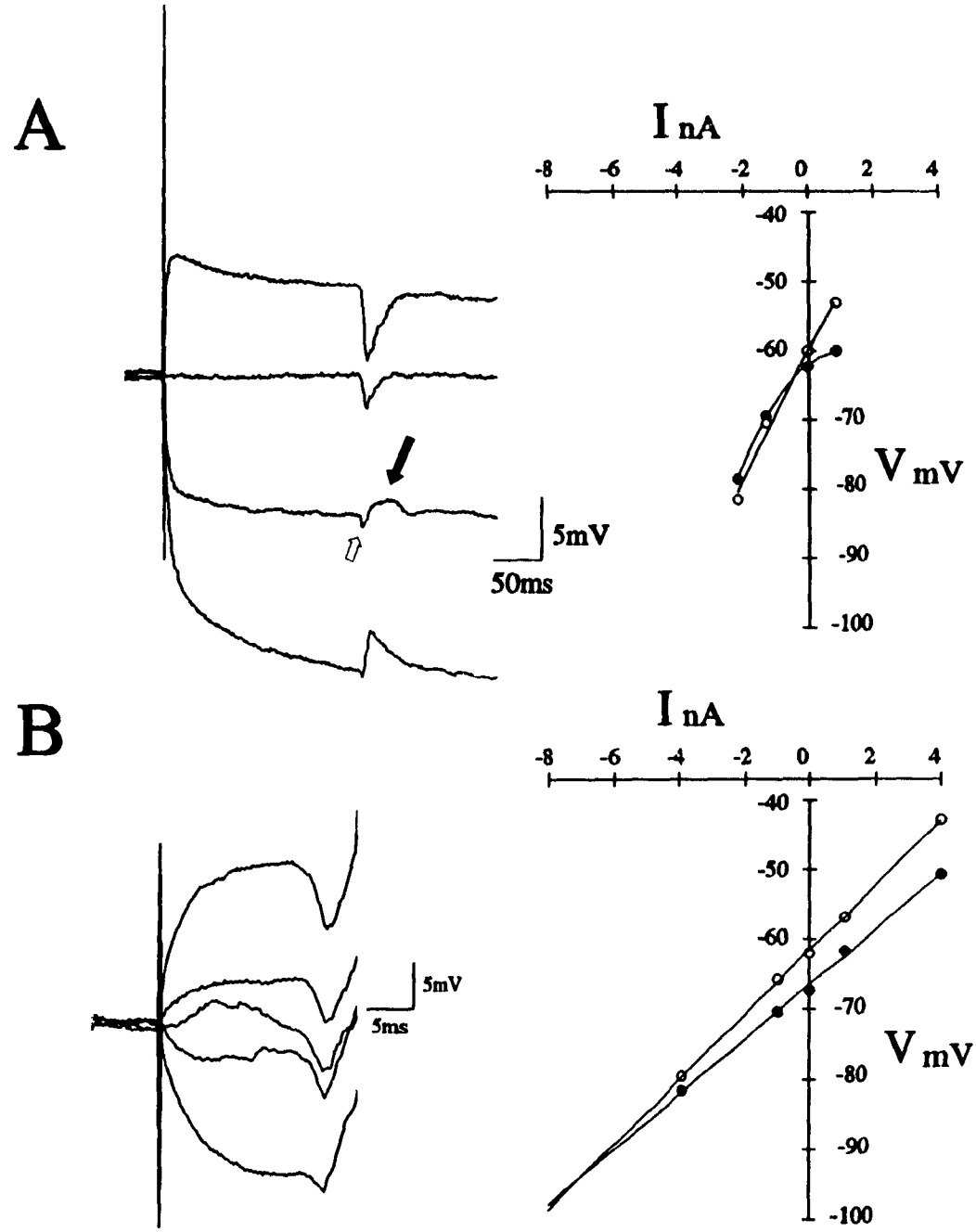

A
I nA
-8
-6
-4
-2
0
2
4
-40
-50
-60
-70
-80
-90
-100
V mV
5mV
50ms
B
I nA
-8
-6
-4
-2
0
2
4
-40
-50
-60
-70
-80
-90
-100
V mV
5mV
5ms

Fig. 6. $GABA_A$- and adenosine-mediated components of the inhibition evoked by pairs of low-threshold mechanical stimuli. Peristimulus time histograms (PSTH) which represent cumulative response to 100 pairs of mechanical stimuli given at 0.2 Hz. The vertical scales represent the number of spikes/bin. The stimuli were applied at the location indicated by the arrow in the diagram. The low-threshold excitatory receptive field of the neurone is represented by the darkened area and the high-threshold excitatory receptive field by the greater hatched area. At a low intensity of stimulation (100 μm; PSTHs on the left), bicuculline (0.6 mg/kg I.V.) blocked the majority of the inhibition (lower PSTH) suggesting that the main component under these conditions is $GABA_A$-mediated (bin width = 5 ms). At a higher intensity of stimulation (1000 μm; PSTHs on the right), bicuculline (0.6 mg/kg I.V.) only partially blocked the inhibition to mechanical stimulation (middle PSTH). Subsequent administration of caffeine (40 mg/kg I.V.) blocked the remainder of the inhibition (lower PSTH) suggesting that the early component of the inhibition is adenosine mediated. (Bin width = 5 ms).

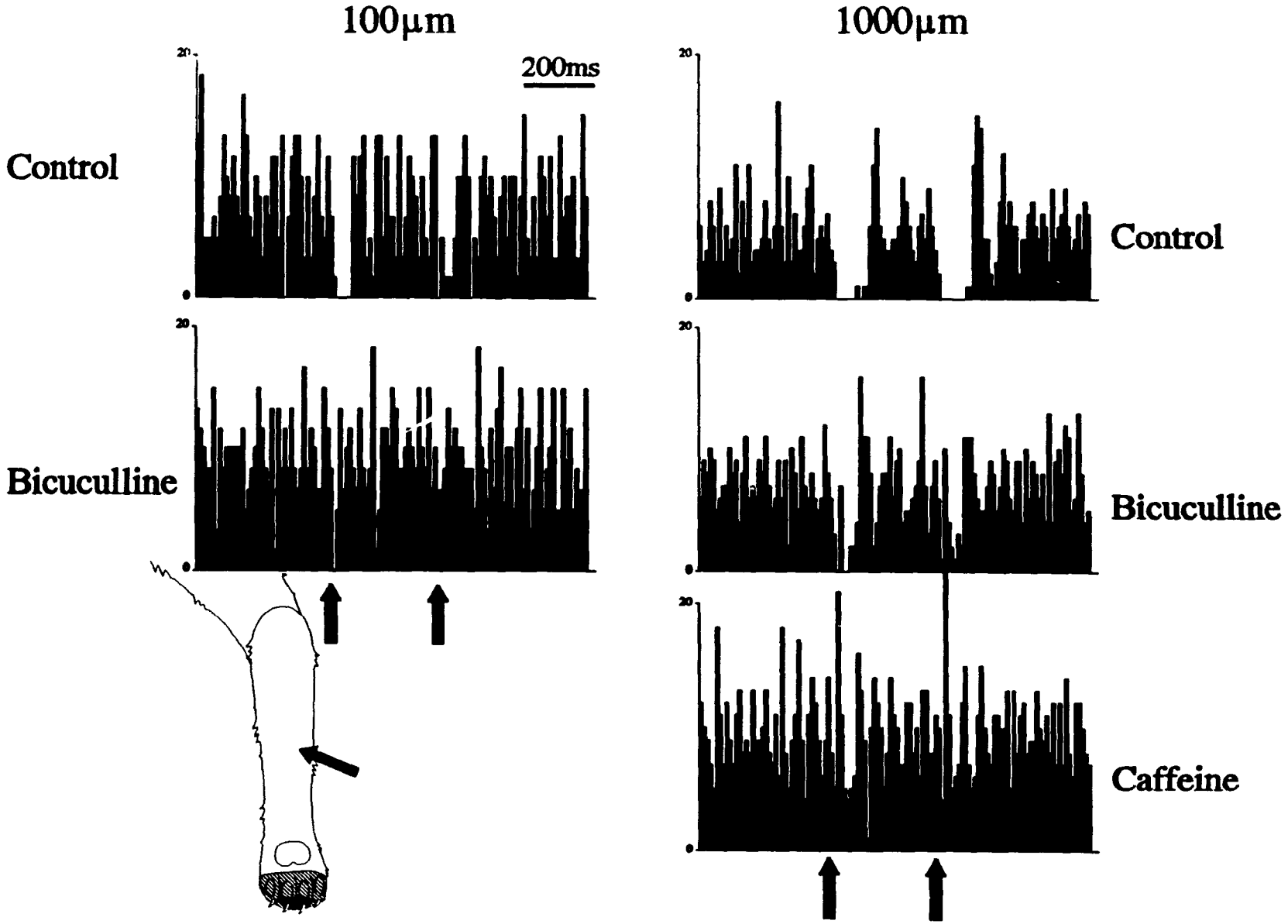

100µm
1000µm
200ms
Control
Bicuculline
Caffeine
20
0

Fig. 7. Early and late components of the inhibition of on-going discharge in response to low-threshold stimulation at different stimulus frequencies. Peristimulus time histograms (PSTH) which represent cumulative response to 50 mechanical stimuli given at 0.5, 1.0 and 2.0 Hz. At a low intensity stimulation, the early and late components were separated by a brief period of excitation. Note that the late component is more depressed at higher frequencies of stimulation (2.0 Hz, lowest PSTH) than the early component. Note also that the later *frequency-sensitive* component represents the majority of the inhibition at lowest frequency (top PSTH on the left). At the higher intensity of stimulation (900 μm), the early component of the inhibition is increased (top PSTH on the right). Note that it is still the late component that is more frequency sensitive (lowest PSTH on the left).

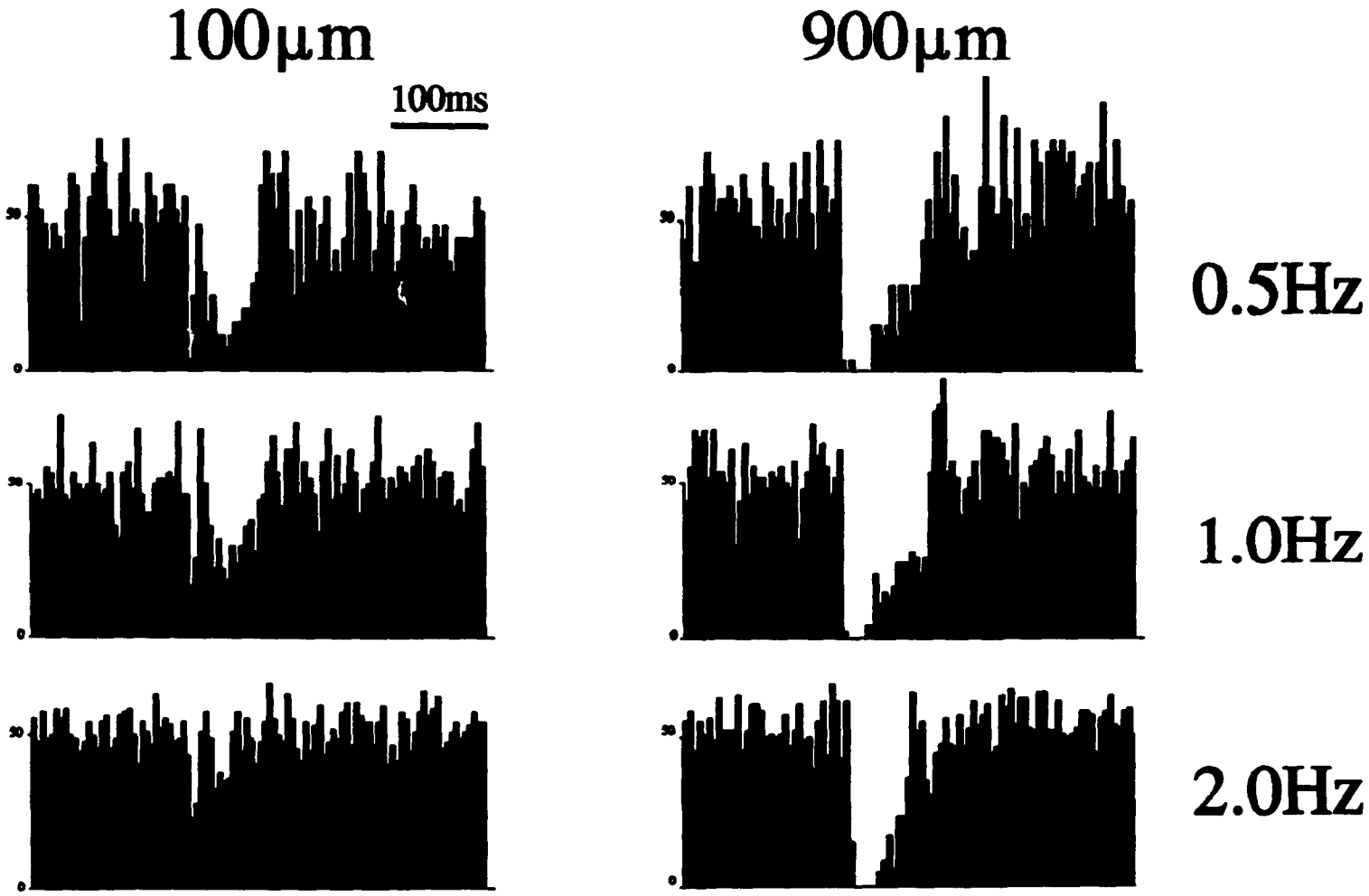
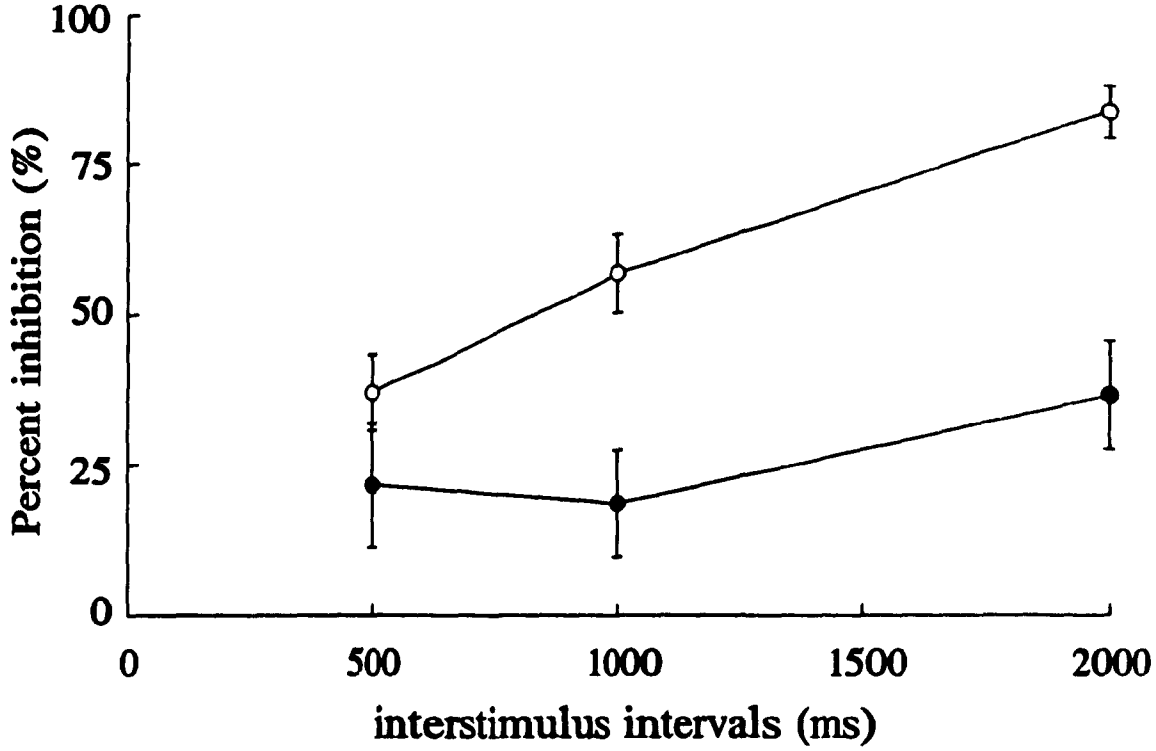
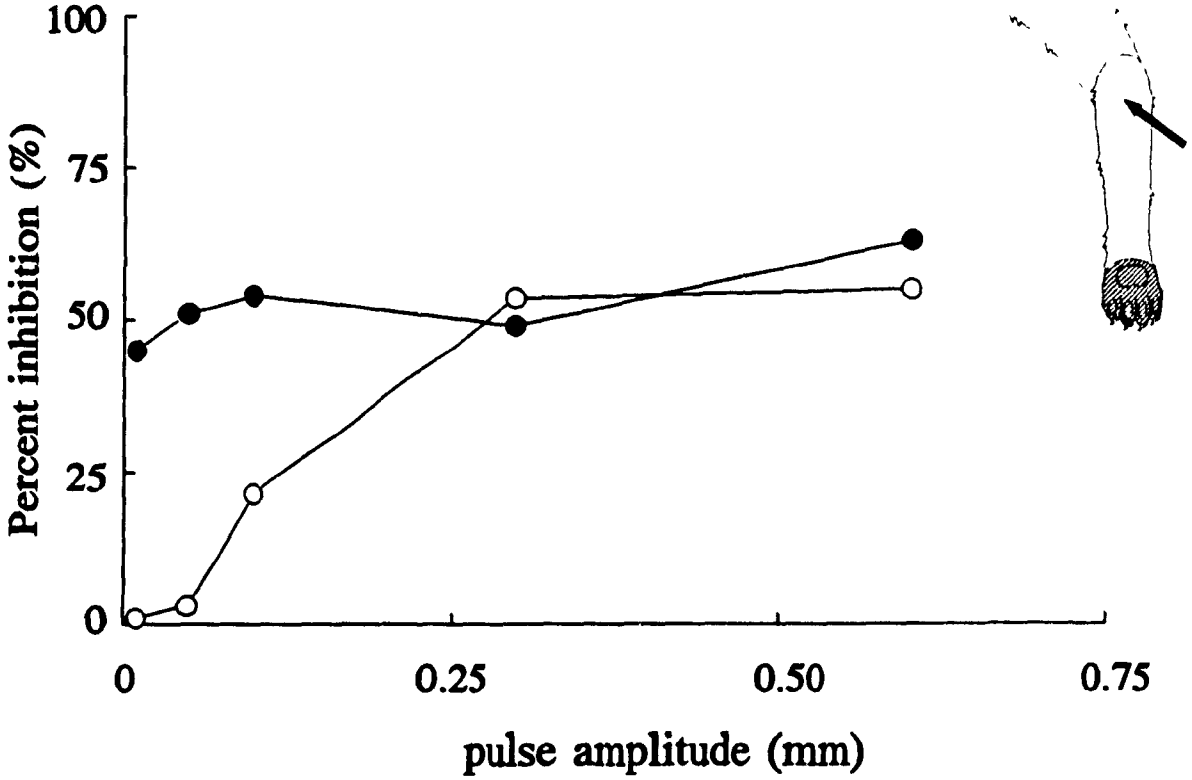

100μm
100ms
900μm
50
0
0.5Hz
1.0Hz
2.0Hz

Fig. 8. Differentiation of the IPSP to the second of paired, low-threshold mechanical stimulation into different components. *A*: Percent inhibition obtained by comparing the average firing in an early ● and a late O window of time following the stimulus, with the average firing prior to the stimulus. The late O, but not the early ● component is significantly depressed at shorter (500 ms) interstimulus interval. *B*: Amplitude response relationship of the overall inhibition (based on the average firing in a window comprising the entire period of inhibition) at 2 different frequencies (● = 0.5 Hz; O = 2.0 Hz) of stimulation. Note the marked inhibition of the response to pulses of small amplitude at 2 Hz frequency of stimulation.


100
75
50
25
0
Percent inhibition (%)
0
500
1000
1500
2000
interstimulus intervals (ms)


100
75
50
25
0
Percent inhibition (%)
0
0.25
0.50
0.75
pulse amplitude (mm)

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CHAPTER V

PROLONGED $GABA_A$-MEDIATED INHIBITION FOLLOWING INPUT FROM SINGLE HAIR AFFERENTS TO SINGLE DORSAL HORN NEURONES IN THE CAT

ABSTRACT

To study the central processing mechanisms of input from low-threshold afferents, we examined the excitatory response of single lamina III-IV dorsal horn neurones to stimulation of the hair receptive field using a mechanical probe and to activation of single hair afferents using an intracellular current pulse to cell bodies in the dorsal root ganglion. The responses to input from the two types of hair afferent were characteristically different. The excitatory response to guard-hair input was multimodal, characterised by a small early component followed by sharp, large component and a slow, prolonged decay phase, whereas that to d-hair input was unimodal. EPSPs from both types of input were attenuated by iontophoretic administration of kynurenate suggesting that they were both mediated by an excitatory amino acid. When the receptive field of the afferent was located in the centre of the receptive field of the dorsal horn neurone, the gain of the central response was markedly greater for the input from a single guard-hair afferent (>1 spike in the dorsal horn neurone per impulse in the afferent) than for the input from a single down-hair afferent (<1 spike in the dorsal horn neurone per impulse in the afferent). The response in the dorsal horn neurone to the second of paired impulses in single guard-hair afferents was markedly depressed at interpulse intervals of less than 2 s, without any apparent post-synaptic inhibition. The response to the second of paired impulses in single down-hair afferents was not as markedly depressed as that do guard-hair input. Administration of bicuculline, but not strychnine or naloxone, attenuated the inhibition of the response to the second of paired guard-hair inputs, suggesting that this inhibition involves a $GABA_A$-mediated mechanism. These results suggest that different classes of afferent give rise to different types of central response and that these inputs are subject to different central regulatory mechanisms.

INTRODUCTION

Brown, *et al.* (1987a) have described a prolonged (>1 s) depression of the excitatory response in dorsal horn neurones to the second of paired inputs from single guard hair (g-hair; group II) afferents. This inhibition appeared not to involve any postsynaptic potential (Brown *et al.*, 1987b). More recently, Koerber & Mendell (1988) studied the behaviour of central responses (cord dorsum field potentials) at varying frequencies of input from a variety of single afferents. They reported variable frequency sensitivity of the input from different types of afferents, some responses increasing in magnitude at higher frequencies of impulses in the afferent, others being unchanged and some being markedly inhibited, in particular the central response to a single g-hair afferent. Their results suggested that the central response to different afferents is subject to different central regulatory controls.

Neither group attempted to elucidate the mechanism of the respective inhibitions. Therefore, to study the possible chemical mechanism by which these inhibitions were elicited, we studied the input from single hair afferents to single dorsal horn neurones using the approach described by Brown *et al.* (1987a). Preliminary results of our study have been published elsewhere (De Koninck and Henry, 1990).

METHODS

Animal preparation

Experiments were done on 25 cats anaesthetized with α-chloralose (60 mg/kg I.V.). The left common carotid artery and the left jugular vein were cannulated and a tracheal cannula was inserted. Spinal segments L_5-S_1 were exposed for recording and the spinal cords were transected at the L_1 vertebral level to eliminate the influence of supraspinal structures. Prior to the spinal transection, to minimize spinal shock, the L_1 segment was injected with lidocaine hydrochloride (Xylocaine 1%, Astra; 0.1 mL). The exposed spinal cord was covered with a pool of warm mineral oil to prevent cooling and drying.

Arterial pressure was monitored continuously throughout the experiment and, when necessary, was maintained above 80 mmHg by intravenous infusion of dextran (Macrodex, Pharmacia; 6% in saline) or noradrenaline bitartrate (Levophed, Winthrop; 0.002% in saline).

After bilateral pneumothorax, the animals were paralysed with pancuronium bromide (Pavulon, Organon; 1 mg/kg I.V.; repeated when required) and ventilated artificially. The level of anaesthesia was confirmed periodically throughout the experiment by examination of the arterial pressure, by checking the degree of pupillary constriction and by allowing the effects of the muscle relaxant to wear off transiently. An additional dose of α-chloralose (30 mg/kg) was given I.V. 7-9 hours after the first injection and beyond that time when required. End tidal CO_2 concentration was

monitored continuously using a Beckman LB2 Medical Gas Analyzer and was maintained between 3.5 and 5.0%. Rectal temperature was maintained at 38°C using a thermistor-controlled servo-mechanism and an infrared bulb.

Recording and activation of single afferents

The L_7 dorsal root ganglion was exposed and an incision made in the connective tissue of the ganglion to expose the cell bodies for intracellular recording (single glass micropipettes; 3 M KCl; 10-20 MΩ). When stable intracellular recording was obtained, intracellular depolarizing pulses were used to activate the ganglion cell.

Simultaneous extracellular recording was obtained from single dorsal horn neurones using multibarrelled micropipettes (recording barrel: 3 M NaCl; 3-4 MΩ). In some cases, glutamate (1M; pH 7.4; Sigma) was applied by iontophoresis to raise the rate of firing of the dorsal horn neurone to enable observation of inhibitions.

In some experiments intracellular recording from dorsal horn neurones was used (single glass micropipettes; 3M $K(CH_3SO_4$; 20-50MΩ) to study responses to mixed hair stimulation of the excitatory receptive.

The micropipettes were mounted on stepping motor drives (Inchworm; Burleigh). The electrodes were connected via locally built DC preamplifiers (PCA-3; electronic workshop, Department of Physiology, McGill University) to a Tektronix 5111 oscilloscope. The amplified raw signal was recorded on magnetic tape using an

Instrutech digitizer (VR-100) and a VCR tape-recorder. The output of the amplifier was also connected to a Data Translation DT2801-A analogue-to-digital board in a personal computer for sampling and analysis of evoked responses from stimulation of the single afferent in the DRG using locally designed software.

In addition, in some experiments the cord dorsum potential was recorded in response to electrical stimulation of afferent nerves and mechanical stimulation of the ipsilateral hind foot. Single glass micropipettes were used (3M NaCl; 1 MΩ). Recording was done with the DC preamplifier, with no high pass filtering.

Intravenous administration of antagonists

Antagonists were administered via the catheter in the external jugular vein. When possible, intravenous administration was preferred to iontophoretic administration to ensure that the antagonist would reach a wide number of receptor sites. The dosages were as follows: bicuculline hydrochloride, 0.3-1.0 mg/kg (Research Biochemicals Inc.) (De Koninck and Henry, 1990; Duggan and Foong, 1985; Salter and Henry, 1988); strychnine sulphate, 0.2-0.6 mg/kg (British Drug Houses); naloxone hydrochloride, 0.1-0.5 mg/kg (Narcan, Endo) (Calvillo *et al.*, 1974, 1979). In experiments where antagonists were administered intravenously, the cats were pretreated with hexamethonium bromide (10 mg/kg I.V. every 5 hours; Sigma) to block the changes in

blood pressure induced by bicuculline (Hong and Henry, 1990) and strychnine (Hong *et al.*, 1989).

Iontophoretic administration of antagonists

In some of the intracellular experiments in the dorsal horn, the single intracellular micropipettes were glued to a multibarrelled micropipette for extracellular iontophoresis (tips separated by approx. 50 μm). The solutions used were kynurenate (20 mM in 100 mM NaCl, pH 7.4, Sigma), glutamate (1 M, pH 7.4, Sigma) and a control solution (100mM NaCl, pH 7.4).

Classes of neurones studied and hair stimulation used

In the present experiment, only guard hair afferents (g-hair; group II) and down-hair afferents (d-hair; group III) were used and only dorsal horn neurones which could respond to movements of a single hair were studied. These neurones were considered to be located in lamina III-IV, based on their physiological properties and based on the depth of the recording. In some experiments, where responses of dorsal horn neurones to mixed hair input were studied, a feedback-controlled mechanical stimulator (Chubbuck, 1966) was positioned on the hair excitatory receptive field such that mechanical displacement would activate only hairs (displacements of the stimulator can range from 1μm-1000μm).

RESULTS

Two types of EPSPs to hair stimulation

Two types of response to hair afferent stimulation were observed during intracellular recording from dorsal horn neurones. Fig. 1 shows the response of a dorsal horn cell to hair stimulation with a mechanical stimulator. This cell was chosen because it illustrates a mixed response to hair stimulation, but it is not representative of the whole population. At low frequencies of stimulation, there was an early response which consisted of a group of EPSPs peaking around 10 ms and slowly decaying over 30 ms and a later small peak at 40 ms. This second response appeared more prominent at higher frequency of stimulation (10-20 Hz), while the early component was depressed. The early component, which occurs at a latency consistent with $A\beta$ afferent conduction (g-hair), is strongly depressed with increasing frequency of stimulation. (Even at the 1 Hz frequency, the early EPSPs are depressed; this frequency was used here to decrease the number of action potentials for clarity of the illustration.) The later EPSP which persists at higher frequencies of stimulation, occurs at a latency consistent with its mediation by afferents in the $A\delta$ conduction range (d-hair). These results hence suggest that the input from g-hair afferents shows a differently sensitivity to the frequency of stimulation than that from d-hair afferents. Note also, in Fig. 1, the characteristic difference in shape of the early (presumed g-hair) EPSP with that of the later (presumed d-hair) EPSP. At a frequency of stimulation of 15 Hz, what appeared as unitary components of the early EPSP (g-hair) were asymmetrical (fast rising with slower decay phase). On the contrary,

the later EPSP (d-hair) was more symmetrical (similar rising and decay phases) and dome shaped. At lower frequency of stimulation, the early EPSP also consisted of a complex of EPSPs suggesting a polysynaptic component. Note also that there is a late IPSP observable after the end of the EPSPs (peak around 50 ms). As this IPSP disappeared at the higher frequency of stimulation, this may account for the increase in size of the late (presumed d-hair) EPSP.

Sensitivity of early and late EPSPs to kynurenate

Fig. 2 shows the effect of iontophoretic application of kynurenate on the EPSPs illustrated in Fig. 1. Both EPSPs are reversibly and repeatably attenuated by extracellular iontophoretic application of kynurenate (also seen with 4 other neurones). The sensitivity of both EPSPs to kynurenate suggests that they are both mediated by activation of an excitatory amino acid receptor.

Responses from impulses in single afferents

To further study each of the different components of the mixed hair input, we looked at input from single hair afferents to single dorsal horn neurones. Eight coupled pairs of single g-hair to single dorsal horn neurones and three coupled pairs of d-hair to single dorsal horn neurones were obtained. Figs. 3 and 4 illustrate responses in dorsal horn neurones from single g-hair and d-hair afferents respectively. Note the similarity

between the response to the single g-hair afferent in Fig. 3 with that of the early EPSP complex at 1 Hz in Fig. 1. and the similarity between the response to the single d-hair afferent in Fig. 4 and the later EPSP in Fig. 1. As was previously observed (Brown *et al.*, 1987c), when the receptive field of the g-hair afferent was contained within that of the dorsal horn neurone, a single action potential in a single g-hair afferent gave rise to more than one spike in the dorsal horn neurone, with the response being greater as the receptive field of the afferent was closer to centre of the receptive field of the dorsal horn neurone. For d-hair afferents, the situation was different. Several impulses in the afferent were necessary to elicit a response in the dorsal horn neurone. This situation was similar to that observed with g-hair afferents when their receptive field of the g-hair afferent was located on the edge of the receptive field of the dorsal horn neurone. However, the shape of the response in the dorsal horn neurones to input from d-hair afferents was, in the three cases studied, characteristically different from that of the response from g-hair afferents regardless of the juxtaposition of the respective receptive fields.

Responses to paired impulses in single afferents

To study the period of inhibition that follows the input from hair afferents, we gave pairs of depolarizing pulses at varying intervals of in the single afferents. Fig. 5 shows the response of a single dorsal horn neurone to pairs of impulses in a single g-hair

afferent. Note the inhibition of the second responses. This inhibition was seen at interpulse intervals of more than 1 s. The same inhibition was observed with pairs of bursts (3-6 spikes separated by 5 ms) in single g-hair afferents and also when bursts of action potentials in the g-hair afferent were necessary to observe a central response (when the receptive field of the afferent was on the edge of that of the dorsal horn neurone). Note also that the tail component of the response to single impulses is more potently depressed than the early component.

The second of paired responses d-hair afferents was however much less depressed than that from g-hair afferents (Fig. 6), suggesting that the input from d-hair is not as sensitive to frequency as that from g-hair. Note also that the background firing activity is not inhibited following the response from the single afferent, suggesting that the inhibition of the second of paired impulses was not due to a postsynaptic mechanism (unless involving a postsynaptic mechanism very specific to the response from the g-hair). This observation is consistent with the observation from Brown *et al.* (1987b), that no prolonged postsynaptic potential was observed that could account for the prolonged (> 1 s) inhibition observed on the second of paired inputs. However, as in Fig. 1, they have observed IPSPs from stimulating a group of hair in the excitatory receptive field of the dorsal horn neurone, but of much shorter duration ($<$ 100 ms). We have also observed IPSPs in dorsal horn neurones to mechanical pulses applied onto the hind foot which could sometimes last up to 400 ms (De Koninck and Henry, 1990; chapter IV).

The present study is however the first in which we observed an inhibition which lasted greater than 1 s.

Comparison of the time course of the inhibition of the response to the second of paired inputs with the time course of the P wave of the cord dorsum potential

Fig. 7 shows a comparison of the time course of the inhibition of the dorsal horn neurone response to the second of paired inputs from single g-hair afferents with the time course of the P wave of the cord dorsum potential (REF). Similar P waves could be obtained from electrical stimulation of afferent nerves and from low-threshold mechanical stimulation (Fig. 7A). This P wave is blocked by bicuculline (0.3-1.0 mg/kg I.V.; n=5 cats; Fig. 7B), suggesting that it reflects the presence of a prolonged (> 1 s) $GABA_A$-mediated mechanism following input from low-threshold peripheral stimulation.

Effect of bicuculline on the inhibition following input from g-hair afferents.

The similarity between the time course of the P wave of the cord dorsum potential and that of the inhibition of paired inputs from g-hair, and the fact that the P wave was blocked by bicuculline prompted us to test whether the inhibition of second of central responses to paired inputs was also affected by bicuculline. Accordingly, bicuculline (0.6 mg/kg; I.V.) reversibly attenuated the inhibition of the second of paired impulses from a single g-hair afferent at 300 ms interpulse interval (n=3; Fig. 8), suggesting that

the prolonged inhibitory period following input from single g-hair afferent involved a central $GABA_A$ mediated mechanism. The same attenuation was observed with the inhibition of the response to the second of paired stimulation of the hair receptive field (mixed hair input; n=5; Fig. 9). Strychnine (n=2) and naloxone (n=3) had no effect on this type of inhibition.

Note, in Fig. 9, a late component of the excitatory response to hair stimulation which appears more prominent with the second of paired stimuli (arrow; top PSTH) while the early component is dramatically depressed. This response resemble that illustrated in Fig. 1, where the late (presumed d-hair) EPSP was emphasized at increased frequencies of stimulation which attenuated the early (presumed g-hair) EPSPs. In Fig. 9, administration of bicuculline (lower PSTH; 1.0 mg/kg, I.V.), while it did not affect the size of the early component of the response, greatly enhanced the late component of the excitation. Bicuculline also attenuated the inhibition of the early component of the response to the second of paired stimulation. Note that, at 250 ms interstimulus interval, whereas the early component was still inhibited in the second of paired responses, the late component was much less inhibited (Fig. 9, lower PSTH). Hence, administration of bicuculline unmasked a late component in the response to mixed hair stimulation. Such unmasking of a late component was not seen with input from single g-hair following bicuculline, suggesting that it is due to activation of a different group of afferents. This late component behaves similarly to the late component of the response described in

Fig. 1, and similarly to the response to input from single d-hair afferent (Fig. 6). Moreover, the latency of this later component is consistent with its mediation by d-hair afferents. These results hence suggest that the late component of the response to mixed hair stimulation in Fig. 9 is mediated by activation of d-hair afferents.

DISCUSSION

In the present study, we have characterized the response of single dorsal horn neurones to activation of single hair afferents. At low frequencies of impulses in the afferent, the shape of the central response to each type of afferent was characteristically different. The response to g-hair input was multimodal, characterised by a small early component, followed by sharp large component and a slow, prolonged decay phase, whereas that to d-hair input was unimodal.

Excitatory mechanisms

The central gain of the response to g-hair input was also greater than that to d-hair input. One possible explanation for this difference may be that the shape of the response to g-hair is due to the addition of a small early monosynaptic component and larger di- and polysynaptic components, as suggested by Brown *et al.* (1987c) and Hongo and Koike (1975); conversely, the response to d-hair input may be mediated more directly via simpler circuitry, for example mainly monosynaptic (and/or disynaptic) components.

This possible difference in the organization of the input of the two types of afferents and the lamina III-IV neurone studied here, may account for the fact that the response to d-hair input is markedly less inhibited than that to the g-hair input at short interstimulus intervals and at higher frequencies of stimulation. Indeed, the later, presumably polysynaptic component of the response to g-hair input appears to be most sensitive to repetitive stimulation (see also Brown *et al.*, 1987b). It is interesting to

speculate whether the different organization of the terminal arborization of these two classes of afferents (flamed shaped arbour for g-hair versus a simpler pattern for d-hair; Light & Perl, 1979; Shortland *et al.*, 1989) may contribute to this physiological difference.

When the response to mixed hair input was reduced to simpler components (probably mono- and disynaptic) by increasing the frequency of stimulation, the shape of the EPSPs still appeared somewhat different, suggesting that perhaps the more direct coupling between the two classes of afferent and the dorsal horn neurone may again differ. Whether this difference is due to the morphological or the chemical nature the different coupling remains to be determined. In this regard, however, the type of excitatory transmitter used by the two types of afferent seems to be the same or closely related as both responses were attenuated in a comparable way by kynurenate and Salt and Hill (1981, 1982) have reported that responses to low-threshold mechanical stimulation is insensitive to NMDA receptor antagonists. The possibility remains, however, that a difference in closely related mechanisms of the non-NMDA type may account for some of the differences in the responses to input from the two types of afferents.

Inhibitory mechanisms

The results of the present study have also demonstrated that a period of inhibition in the dorsal horn following input from hair afferents is mediated via a $GABA_A$ mechanism. As mentioned above, this inhibition appears most prominent with g-hair

input, possibly due to the larger polysynaptic component of its central response; the latter component of the excitatory response and its depression are also discussed in Brown *et al.* (1987a) and Koerber and Mendell (1988).

IPSPs in dorsal horn neurones following low-threshold input have been described previously (Hongo *et al.*, 1966, 1968; Brown *et al.*, 1987b; chapter IV). The duration of the inhibition described above is longer than that of these IPSPs. Interestingly, in the present study, inhibition of the on-going firing activity of a dorsal horn neurone was never seen following input from a single hair afferent. However, hair stimulation in the excitatory receptive gives rise to IPSPs (Fig. 1; Hongo *et al.*, 1968; Brown *et al.*, 1987b). As bicuculline did not change the shape of the decay phase of the responses from single afferents, it is unlikely that the IPSP would be hidden in the decay phase of the excitatory response. It is possible that the mechanism giving rise to IPSPs triggered from activity in a single afferent is too small to be observed in the present conditions. Another possibility is that the IPSP arises from activation of a different class of afferents. Nevertheless, the inhibition described here outlasts the duration of previously described $GABA_A$-mediated IPSPs (chapter IV) in dorsal horn neurones. Hence if an early portion of the response may be postsynaptic, an important component probably involves mechanisms presynaptic to the dorsal horn neurones studied.

The fact that the P wave of the cord dorsum potential is blocked by bicuculline indicates that the pronounced the pronounced GABAergic mechanism distributes widely

throughout the spinal cord following input from a single peripheral stimulus. This P wave has been suggested to reflect primary afferent depolarization (see Willis and Coggeshall, 1978). While the time course of PAD is usually of shorter duration than the time course observed here, a number of the earlier studies have used AC coupling which may truncate the late phase of the slow wave of PAD. This difference in the recording may account for some of the discrepancy. Whether the P wave reflects PAD or not, it nevertheless reflects the prolonged GABAergic system triggered from low-threshold input. This GABAergic system appears to participate in central changes occurring with the arrival of g-hair input and serves to attenuate the *impact* of this input upon repetitive activation.

Interestingly, whereas the effectiveness of a single g-hair input seems much stronger in eliciting a central response than does a single d-hair input, the effectiveness the g-hair input drops dramatically with repetitive stimulation which is not the case with the d-hair input (see also Brown *et al.*, 1987c; Koerber and Mendell, 1988). One consequence of the characteristics of each of these inputs is that, upon mechanical stimulation of the hair receptive field, as the response to d-hair input occurs at a later latency (Aδ conduction velocity) than does the g-hair input (Aβ conduction velocity), at low frequencies of stimulation, the d-hair response is masked by the period of inhibition following the early g-hair response (Brown *et al.*, 1987b; Noble and Short, 1989; Short *et al.*, 1990). However, as the frequency of stimulation is increased and the response to

g-hair input is attenuated, the d-hair response becomes a more significant component of the overall response.

These results suggest that the two hair inputs discussed in the present study are subject to different central regulatory mechanisms which may reflect different central encoding of the information mediated by each of these inputs.

Fig. 1. Response of a dorsal horn neurone to hair stimulation in the excitatory receptive field. Each set of traces represents 5 superimposed traces of the response to a brief mechanical pulse from a mechanical stimulator apposed onto hairs in the excitatory receptive field (see inset). The displacement of the mechanical stimulator was restricted to hairs and did not make contact with the skin. Note that, at 1 Hz stimulation (*upper traces*), the large early EPSP complex peaking at 7 ms (latency of onset = 7 ms; giving rise to an action potential at 10 ms) and the smaller EPSP peaking at 40 ms (latency to onset = 23 ms). If the two sets of EPSPs correspond to activation of two different populations of afferent fibres, the first one would correspond to a conduction velocity in the 60 m/s range (consistent with Aβ range, g-hair) and the later one would correspond to a conduction velocity in the 15 m/s range (consistent with Aδ range, down-hair). Note also the marked reduction of the early response as opposed to the later response at frequency of stimulation of 15 Hz (*middle traces*). At a lower intensity of stimulation (50 μm), only the later EPSP was observed suggesting that the threshold for the later EPSP was lower than that of the early EPSP and that these two groups of EPSPs result from activation of different afferents fibres. Recording with a $K(CH_3SO_4)$ filled electrode.

Fig. 2. Iontophoretic application of kynurenate attenuates both EPSPs to hair stimulation. Recording was from the same cell as in Fig. 1. Each trace represents an average of the responses to 20 mechanical stimuli to the hair receptive field (stimulation at 15 Hz). Kynurenate (Kyn) was applied twice by iontophoresis using 100 (1^{st}) and then 200 nA for 1 min. The attenuation of the EPSPs slowly increase over during the application and slowly decayed after the end of the application over 1 min. No change in the membrane potential was observed. Current injection through a control barrel containing NaCl had no effect on the membrane potential or the size of the EPSP. Records showing attenuation were taken at the end of each application of kynurenate and those showing recovery were taken 5 min after the end of each application. Recording with a $K(CH_3SO_4)$ filled electrode.

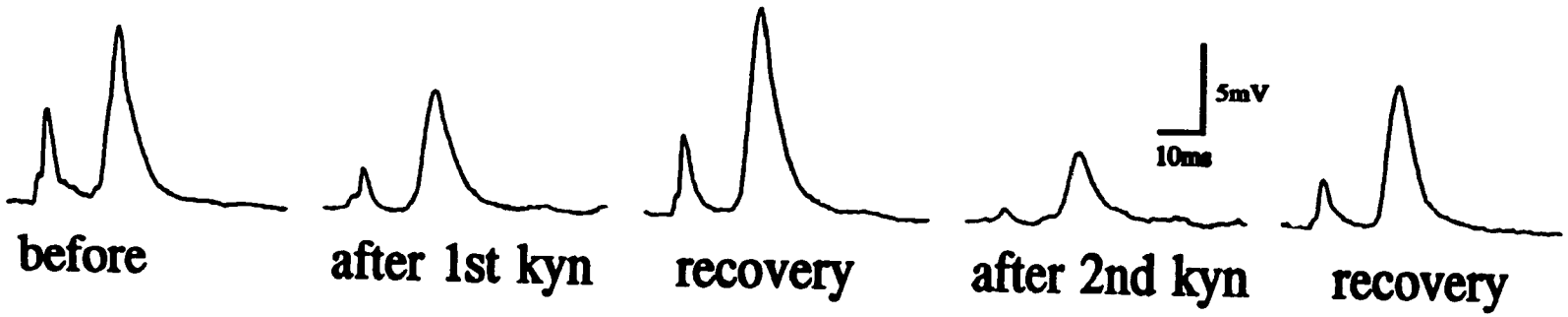

5mV
10ms
before
after 1st kyn
recovery
after 2nd kyn
recovery

Fig. 3. Response of a single dorsal horn neurone to activation of a single g-hair afferent. The records on the left are 15 superimposed traces of the extracellular response of the dorsal horn neurone (*upper*) to impulses in a single g-hair afferent produced by intracellular depolarizing current pulses in the cell body in the dorsal root ganglion (*lower*). The schematic diagram on the right represents the receptive fields of the dorsal horn neurone (DH) and of the dorsal root ganglion afferent (DRG). The peri-stimulus time histogram represents the cumulative response of the dorsal horn neurone (in spikes per bin) to 250 impulses in the single afferent delivered every 3 s (arrow; bin width = 1 ms). The time scale is the same for the records on the left and the histogram.

Fig. 4. Response of a single dorsal horn neurone to activation of a single d-hair afferent. The records on the left are 15 superimposed traces of the extracellular response of the dorsal horn neurone (*upper*) to trains of six impulses in a single g-hair afferent produced by intracellular depolarizing pulses in the cell body in the dorsal root ganglion (*lower*). The schematic diagram on the right represents the receptive fields of the dorsal horn neurone (DH) and of the dorsal root ganglion afferent (DRG). The peri-stimulus time histogram represents the cumulative response of the dorsal horn neurone (in spikes per bin) to 250 trains of impulses in the single afferent delivered every 3 s (arrows; bin width = 1 ms). The time scale is the same for the records on the left and the histogram.

Fig. 5 Response of a single dorsal horn neurone to pairs of intracellular current pulses at different interpulse intervals to a single g-hair afferent. Each peristimulus histogram (bin width = 2 ms) is cumulative of the extracellular response of the dorsal horn neurone to 100 pairs of inputs from the afferent. The interpulse intervals are, from top to bottom, 750, 500, 175 and 50 ms. Arrows indicate the time of occurrence of the current pulse to the hair afferent. Note, in each histogram, the inhibition of the second of paired responses in the dorsal horn neurone. Even at intervals of 1.5 s, an inhibition of the second response was observed (not shown). Note also that the late phase of the excitatory response to the conditioning stimulus is absent in the second response of each pair. The schematic diagram represents the locations of the receptive fields of the g-hair afferent (black) and the excitatory receptive field of the dorsal horn neurone (hatched area).

Fig. 6. Comparison of the inhibition of the response in the dorsal horn neurone to the second of paired inputs from a single g-hair afferent the response in a different dorsal horn to the second of paired input from single d-hair afferent in the same cat. Points on the graph represent means ± SEM (n=100) of the number of spikes in a 30 ms window of the second of paired excitatory responses from single afferent activation normalised according to the size of conditioning response (value at 3 s interval). The peri-stimulus time histograms illustrate the phenomenon represented by the points on the graph (arrows; bin width 1 ms; average responses from 100 pairs of impulses in single afferent). Note that the d-hair input is weaker than that of the g-hair, but is much less inhibited at lower interstimulus intervals than the latter.

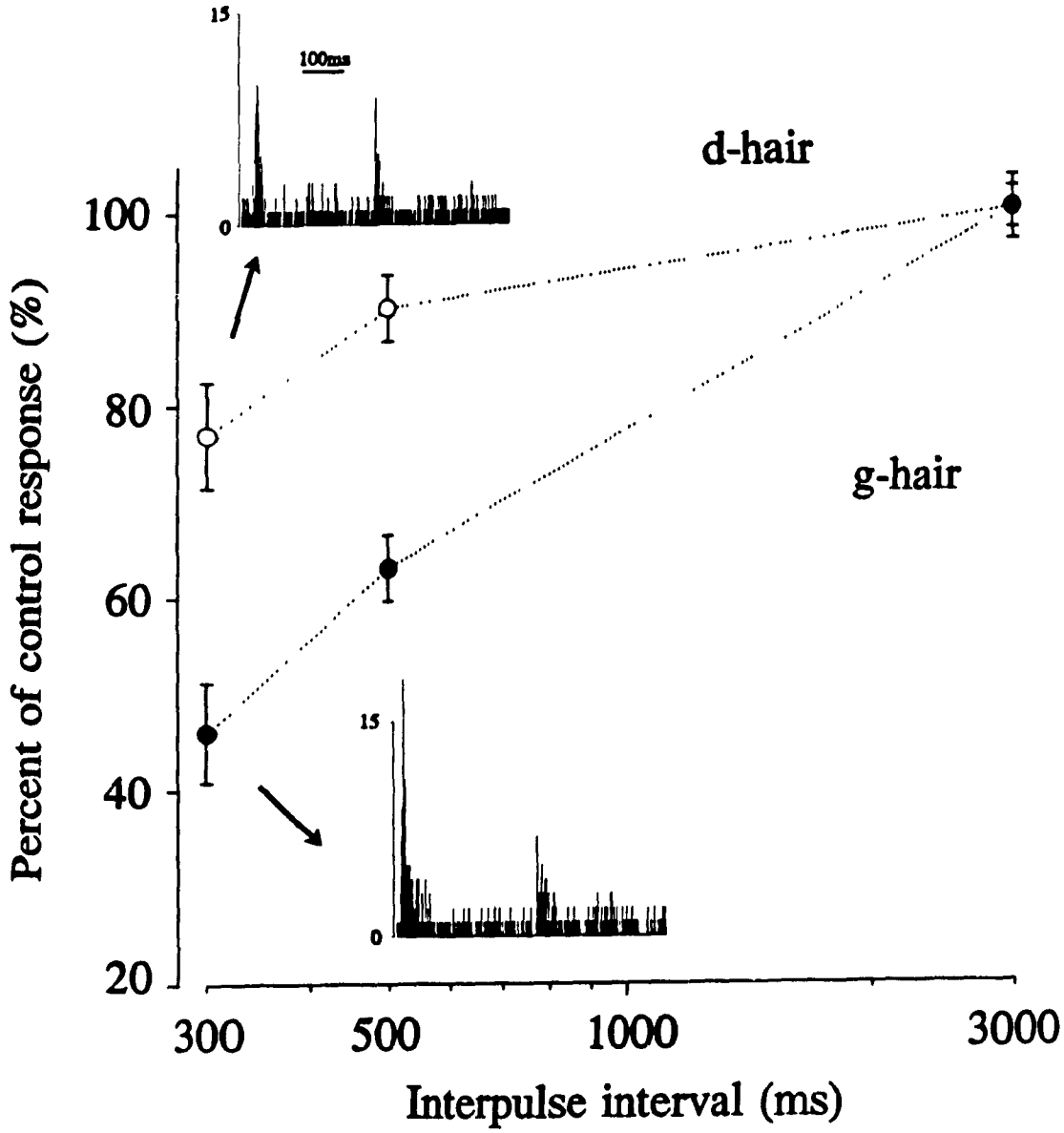

15
100ms
0
d-hair
g-hair
15
0
100
80
60
40
20
Percent of control response (%)
300
500
1000
3000
Interpulse interval (ms)

Fig. 7. Time course and pharmacology of the P wave of the cord dorsum potential. **A:** P waves recorded following electrical stimulation of the sciatic nerve (*left*) or mechanical stimulation of the ipsilateral hind foot (*right*). Each trace is an average of responses from 35 stimuli (50, 60, 80, 100 and 300μA respectively for electrical stimulation; 5, 10, 30, 50, 100μm for mechanical stimulation; stimulation rate 0.1 Hz). Note the prolonged time course of the P wave (> 1 s in duration). **B:** Bicuculline (Bic; 0.6 mg/kg I.V. reversibly blocked the P wave. Each trace is an average of 35 responses from electrical stimulation of the sciatic nerve (75μA at 0.1 Hz). **C:** Comparison of the time course of the P wave with that of the inhibition of the second of paired inputs from a single g-hair afferent to a single dorsal horn neurone in the same cat. The trace at the top of the graph is an average of cord dorsum potentials from 35 stimuli (75μA at 0.1 Hz) to the sciatic nerve. Note the similarity between the time course of the inhibition of the second of paired inputs from a single guard afferent and that of the P wave.

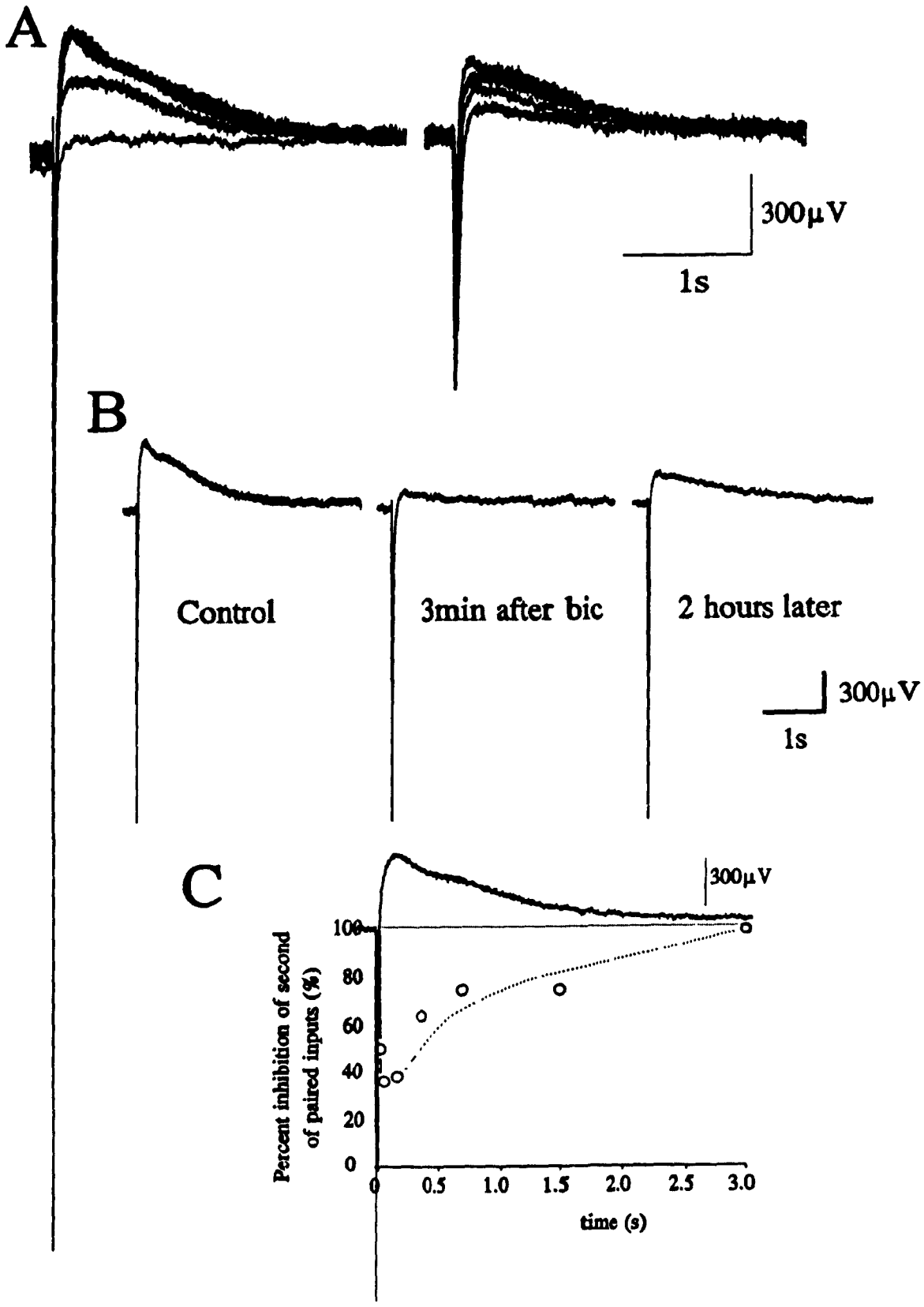

A
300μV
1s
B
Control
3min after bic
2 hours later
300μV
1s
C
300μV
Percent inhibition of second of paired inputs (%)
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
time (s)

Fig. 8. Bicuculline blocks the inhibition of the second of paired inputs from single g-hair afferents to single dorsal horn neurones. The vertical bars represent mean ± SEM (n = 100) of the second of paired excitatory responses to two impulses (interpulse interval = 250 ms) in a single g-hair afferent with respect to the size of the response to the first impulse (100%). Samples taken before and 5 min after each administration of bicuculline (Bic; 0.6 mg/kg; I.V.). Bicuculline attenuated in a dose dependant manner the inhibition of the response to the second of paired impulses, but did not affect the size of the response to the first impulse (not shown).

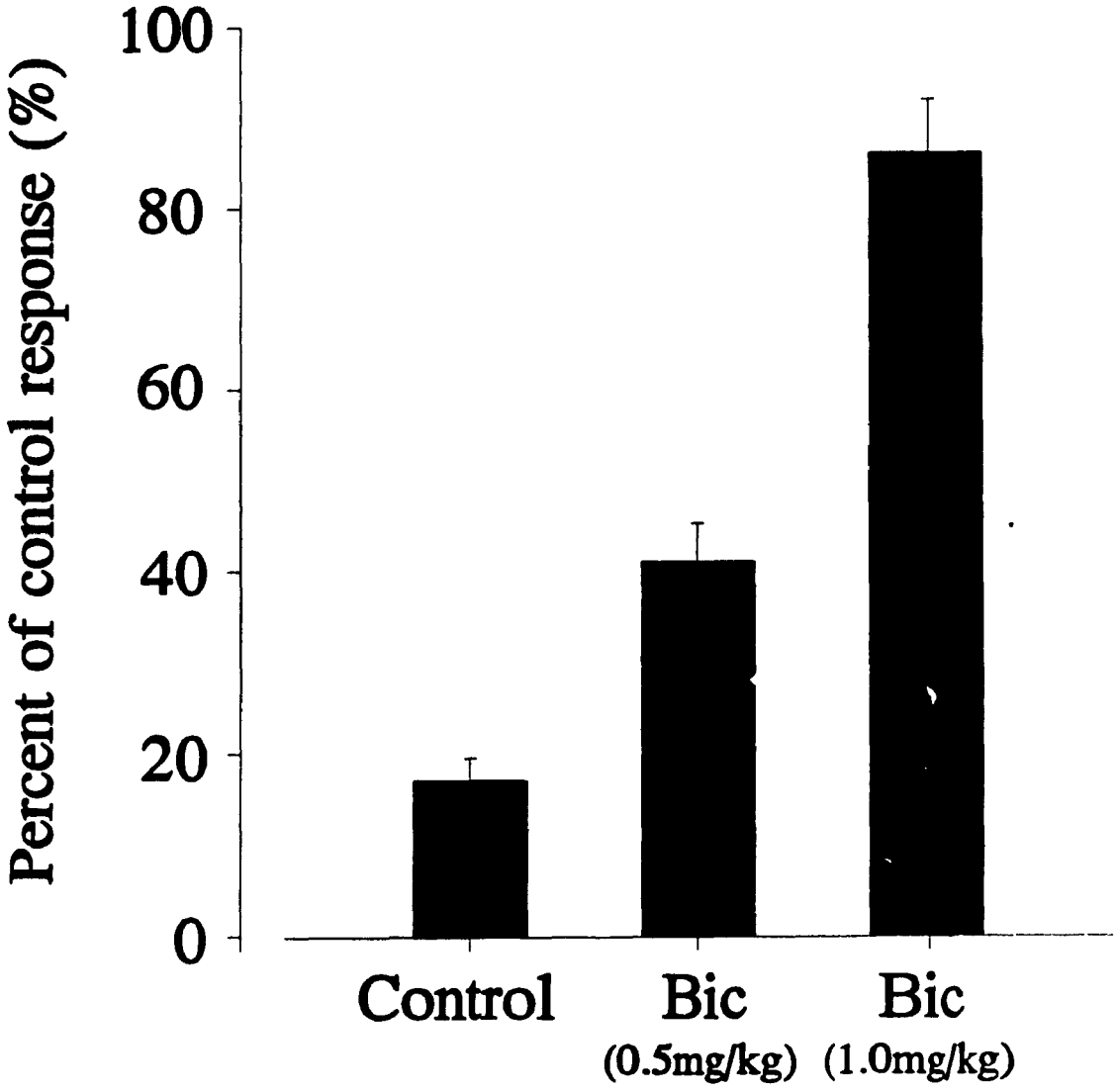
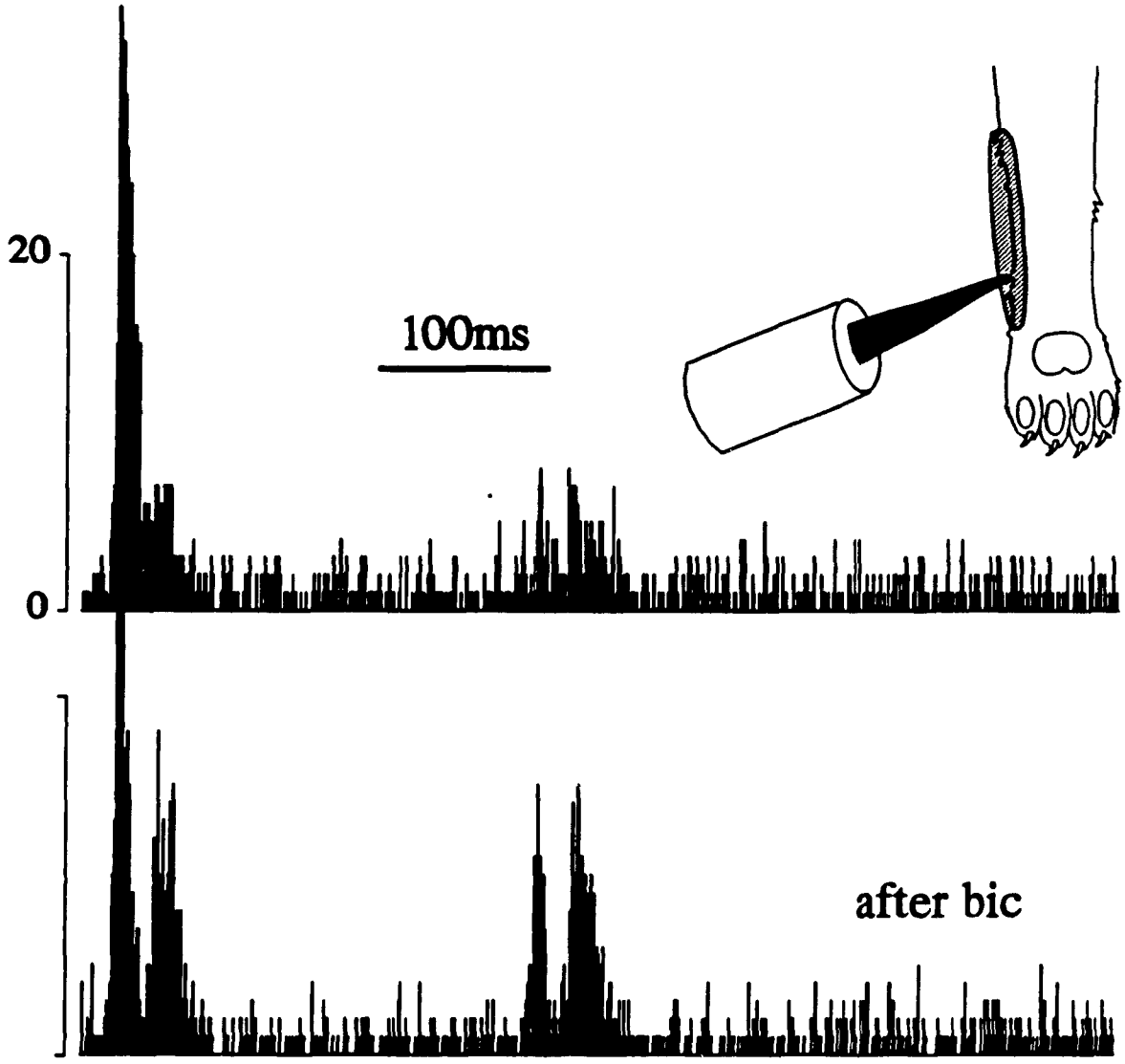

Percent of control response (%)
100
80
60
40
20
0
Control
Bic
(0.5mg/kg)
Bic
(1.0mg/kg)

Fig. 9. Response of a single dorsal horn neurone to pairs of mixed inputs from hair stimulation in the excitatory receptive field. Peri-stimulus time histograms (PSTH) are averages of responses in the dorsal horn neurone to 150 pairs of mechanical pulses moving hair (bin width = 1 ms; tne displacement of the probe did not touch the skin). Note, on the top PSTH, the late component to hair stimulation which appears more important with the second stimulus (arrow) whereas the early response is dramatically depressed. Bicuculline (Bic; lower PSTH; 1.0 mg/kg, I.V.) attenuated the inhibition of the early component of the excitation and unmasked the late component of the excitation. Note that the late component does not appear to be inhibited at this interstimulus interval which contrasts with the early component.


20
0
100ms
after bic

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CHAPTER VI

SUBSTANCE P-MEDIATED SLOW EPSP ELICITED IN DORSAL HORN NEURONS *IN VIVO* BY NOXIOUS STIMULATION

ABSTRACT

The original proposal that substance P is involved in the regulation of nociceptive information at the first sensory synapse in the spinal cord has been substantiated by a wide range of evidence, but definitive support has been lacking primarily because nociceptive input to the dorsal horn has not been blocked by a specific substance P antagonist. Here, we present evidence that CP-96,345, a novel specific substance P (NK-1) receptor antagonist, selectively blocks a slow, prolonged EPSP following noxious cutaneous stimulation or a train of intense electrical stimuli to sensory nerves. These results indicate the specific involvement of substance P in the mediation of a prolonged after-excitation to noxious stimulation. This may have important implications for chronic pain and for plastic changes in nociceptive pathways.

Substance P has been proposed to regulate nociceptive information at the first sensory synapse in the spinal cord (Henry, 1976), on the basis that it is found in small diameter sensory fibers (Hökfelt *et al.*, 1975a, 1975b) which presumably mediate nociceptive inputs (Torebjork and Hallin, 1973; Torebjork, 1974) and on the basis that it specifically excites nociceptive neurons in this region (Henry, 1976). This proposal has been substantiated by subsequent evidence. Nerve terminals containing substance P and substance P receptors are concentrated in regions of the dorsal horn where nociception is integrated (Hökfelt *et al.*, 1975b, 1977; Cuello *et al.*, 1977; Quirion *et al.*, 1983; Ruda *et al.*, 1986). Levels of substance P in these regions decrease when sensory roots are cut or compressed (Takahashi and Otsuka, 1975; Hökfelt *et al.*, 1975a, 1975b). Substance P is released in the spinal cord *in vivo* by activation of nociceptive primary sensory fibers (Theriault *et al.*, 1979; Broding *et al.*, 1987; Go and Yaksh, 1987; Duggan *et al.*, 1987, 1988; Cridland and Henry, 1988) and release is blocked by administration of morphine and opioid peptides *in vivo* and *in vitro* (Jessell and Iversen, 1977; Yaksh *et al.*, 1980; Go and Yaksh, 1987). Finally, analgesia is observed in animals following depletion of substance P from sensory fibers by capsaicin (Yaksh *et al.*, 1979) and patients with diminished pain sensitivity in familial dysautonomia have decreased levels of substance P in sensory fibers (Pearson *et al.*, 1982).

Despite this seemingly convincing evidence, definitive support for the original hypothesis has been lacking, due primarily to the lack of evidence that a nociceptive response in the dorsal horn is blocked by a substance P antagonist. Heretofore, an effective substance P antagonist has not been available. Such an antagonist, CP-96,345, highly selective for the Substance P (NK-1) receptor, has recently been discovered (Snider *et al.*, 1991; McLean *et al.*, 1991) and was made available to us by Pfizer Central Research (Groton, Conn.). Thus, to test its efficacy against nociceptive and C-fiber-induced effects, we recorded intracellularly the prolonged responses of cat dorsal horn neurons *in vivo* to noxious mechanical stimulation of the cutaneous excitatory receptive field as well as to electrical stimulation of sensory nerves.

Cats were anaesthetized with α-chloralose (60 mg/kg i.v.) or decerebrated at the inter-collicular level after induction with halothane (Fluothane, Hoechst). Spinal segments L_5-L_7 were exposed for recording and the cords were transected at the L_1 vertebral level. The cats were paralysed with pancuroniumbromide (Pavulon, Organon; 1 mg/kg i.v.) and ventilated artificially. End tidal CO_2 concentration was maintained between 4.0 and 5.0%. Rectal temperature was maintained at 38°C. Single glass micropipettes were used for the recording (3 M KCl, 2 M K-Acetate or 2 M $K(CH_3SO_4)$; resistances 10-50 MΩ). Criteria for a good penetration were as follows: a negative shift in DC potential of at least 40-50 mV upon penetration, a recovery from injury spiking within a few seconds, a stable resting membrane potential of -50 to -65 mV and spikes with overshoot. The

natural stimuli used were movement of hair manually (or with a feedback-controlled mechanical stimulator (Chubbuck, 1966), brief and also sustained innocuous touch/pressure and noxious pinch of the skin with a serrated forceps at an intensity which we have found to recruit nociceptors (by recording from single dorsal root ganglion neurons). CP-96,345 (Pfizer Inc., Groton, U.S.A.) was dissolved in saline and was slowly (over a 2-5 min period) administered I.V. at doses of 0.5-2.0 mg/kg. Intravenous injection was preferred to local administration to ensure that the antagonists would reach all the receptor sites.

The present study included only wide dynamic range (WDR) neurons because not only are they clearly implicated in the integration of nociceptive information (Price and Dubner, 1977) but they also respond differently to noxious and to innocuous cutaneous stimuli in that noxious but not innocuous stimuli provoke a prolonged after-excitation following the end of the stimulus (Fig. 1) (De Koninck and Henry, 1989; Salter and Henry, 1985). This prolonged response, which can last from 20 s to 10 min, is thus considered to represent a specific nociceptive response and can be mimicked by electrical stimulation of sensory fibers, but only at intensities which recruits C-fibers (Fig. 4Bb). Thus we were able to compare responses to both noxious and innocuous inputs in the same neuron.

The importance of focusing on the slow, prolonged excitation (Fig. 1) as the possible response mediated by substance P is further highlighted by the fact that

stimulation of both low and high threshold afferents evokes EPSPs that are relatively short in duration (in the order of milliseconds; *e.g.* see Fig. 4). They do not correspond to the typically slow excitation observed with substance P on sensory neurons *in vivo* (Henry *et al.*, 1975, 1980; Henry, 1976; Randić and Miletić, 1977) and *in vitro* (Nowak and Macdonald, 1981, 1982; Murase *et al.*, 1982; Murase and Randić, 1984). In addition, Aδ- and C-fiber evoked EPSPs from single stimuli can be antagonized by excitatory amino acid antagonists (Schneider and Perl, 1988; Yoshimura and Jessell, 1990). These observations thus raise the question of what type of natural stimulus evokes the release of endogenous substance P centrally.

Detailed observation of the intensity/response relationship revealed that while the magnitude of the fast depolarization and the number of action potentials recorded during a stimulus train to the sensory nerve reached the maximum at an intensity which recruits Aβ fibers, after-excitation and slow depolarization following the train are preferentially associated with higher intensities which recruit the small diameter C-fibers (2 neurons recorded intracellularly and 3 extracellularly; *e.g.* see Fig. 4B). The after-excitation could also be potentiated by block of large diameter myelinated fibers. Anodal block was applied proximal to the stimulating electrode (Sassen and Zimmermann, 1973) on the superficial peroneal nerve. The intensity of the DC current was sufficient to block the early Aβ latency response. With 2 cells recorded extracellularly, anodal block potentiated both the C-fiber latency response and the after-excitation following train

stimulation of the afferent nerve (not shown). The latter result thus confirmed the predominant participation of small diameter afferents in the mediation of this after-excitation.

Hence, a slow depolarization similar to the one observed following a sustained noxious mechanical stimulus (Fig. 1) is also observed following a train of high intensity electrical stimuli to the sensory nerve (Figs. 2 and 3), suggesting that sustained activation of nociceptive afferent fibers may be necessary to provoke this after-excitation and that it is not due to a peripheral injury, but to a central mechanism. Hence, the similarity between the response to noxious stimulation and that to electrical stimulation of sensory nerves allowed us to use the train of electrical stimuli as a controlled stimulus to produce the slow depolarization (Fig. 4).

This slow depolarization is typically associated with an apparent increase in membrane resistance (Fig. 2), as has been observed with the effects of exogenous application of substance P under current clamp conditions *in vivo* and *in vitro* (Krnjević, 1991; Nowak and Macdonald, 1981, 1982; Murase *et al.*, 1982; Murase and Randić, 1984). This increase in input resistance contrasts with the marked decrease in resistance observed during the train, which is presumably due to a barrage of fast EPSPs. A decrease in the amplitude of the slow depolarization was observed at hyperpolarized potentials and an increase was observed at depolarized potentials (Fig 3B), as has been reported with exogenous substance P application *in vitro* (Nowak and Macdonald, 1981,

1982; Murase *et al.*, 1982; Murase and Randić, 1984). On the other hand, when a single electrical stimulus was given instead of a train, the C-latency EPSPs recorded had a reversal potential comparable to more classical EPSPs, such as those of the non-NMDA type; this is consistent with earlier observations *in vitro* that the Aδ- and C-fiber evoked EPSPs from single stimuli can be antagonized by excitatory amino acid antagonists (Schneider and Perl, 1988; Yoshimura and Jessell, 1990).

As the electrical properties of the late slow EPSP are consistent with the involvement of substance P receptor activation, we determined the effects of CP-96,345 on this slow EPSP evoked by noxious mechanical stimulation of the skin or a high intensity train of electrical stimuli to the sensory nerve. In the four cells tested, CP-96,345 blocked the slow after-excitation following both types of stimulation. This effect was reversible, repeatable and varied with the dose of CP-96,345. At higher doses, a transient decrease in blood pressure was observed. However, antagonism of the slow EPSP was seen at doses that did not provoke any change in blood pressure. Importantly, this effect of CP-96,345 was selective because it did not affect the brisk response to innocuous hair movement. CP-96,345 also did not block the EPSPs recorded at C-latency in response to a single electrical stimulus. Nor did CP-96,345 block the excitation during the train of electrical stimuli to the sensory nerve or the excitation seen during the noxious stimulus (Fig. 4). The specific blockade of the late, prolonged EPSP observed in response to noxious cutaneous stimulation and to repetitive impulses in high threshold

C-fiber afferents suggests that this EPSP is mediated via the activation of substance P (NK-1) receptors. This result suggests that sustained activation of nociceptive sensory fibers provokes the release of endogenous substance P in the dorsal horn.

The idea that substance P may be released preferentially as a result of a sustained noxious stimulus, which can be mimicked by a train of high intensity stimulation to the afferent nerves, is consistent with the description of a substance P-mediated slow EPSP in rat spinal cord slices following train stimulation of the dorsal rootlets (Urbán and Randić, 1984; Randić *et al.*, 1986). Preferential release with repetitive stimulation has been reported for other peptides in peripheral tissues (Lundberg and Hökfelt, 1983).

One should note in this context that the response to a single impulse in C-fibers was not mediated via NK-1 receptors. This is of particular importance as it suggests that the release of substance P may occur only in response to a specific pattern of firing in nociceptors. The first possibility that comes to mind for the source of substance P is that it is released from primary afferents. This hypothesis, together with the fact that the C-fiber induced EPSP (which is not blocked by CP-96,345) in response to a single stimulus appears to be mediated by something else than substance P, possibly an excitatory amino acid (Schneider and Perl, 1988; Yoshimura and Jessell, 1990; see Figs. 3 and 4), would suggest the presence of both substance P and an excitatory amino acid in the same primary afferent. In fact, immunocytochemical studies indicate the colocalization of substance P and glutamate in the same sensory neurons (De Biasi and

Rustioni, 1988; Battaglia and Rustioni, 1988). Another source of substance P could be from descending fibers. However, the after-excitation to noxious and high intensity electrical stimulation was observed in spinalized cats ruling out the possibility of a supraspinal loop in the mediation of this response. The substance P may also have originated, at least partially, from intrinsic neurons in the dorsal horn.

NMDA receptor antagonists block the nociceptive response of motoneurons but not of dorsal horn neurons (Headley *et al.*, 1987). These antagonists also block the facilitation of the tail-flick reflex produced by both substance P and noxious tail immersion (Yashpal *et al.*, 1991). In addition, substance P antagonists block the nociceptive response of motoneurons (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1985; Otsuka and Yanagisawa, 1988) and the facilitation of the tail-flick reflex produced by both substance P and tail immersion (Yashpal and Henry, 1984; Cridland and Henry, 1988). These observations thus suggest that the predominant site of action of substance P in mediating a period of increased excitability following noxious input may be in the dorsal horn. This fully supports the original proposal that substance P acts as a regulator of excitability in nociceptive pathways at the first sensory synapse (Henry, 1976), perhaps by increasing the sensitivity of nociceptive dorsal horn neurons to all synaptic inputs (Salter and Henry, 1987, 1988; Randić *et al.*, 1990).

Our results suggest a role for substance P in the mediation of a prolonged excitation of nociceptive neurons in the dorsal horn following noxious peripheral

stimulation rather than mediating the *fast* EPSP from high threshold afferents. This is consistent with the observation that previous substance P antagonists do not affect the nociceptive reflex *per se*, but rather the potentiation of this reflex which follows prolonged noxious stimulation (Cridland and Henry, 1988). This prolonged excitation may represent the neuronal substrate underlying central components of hyperalgesia. In addition, the control of substance P transmission may be particularly useful in the management of at least some types of chronic, nociceptive pain as well as to the modification of long term changes in nociceptive pathways following noxious stimulation (Woolf, 1983; Woolf and King, 1990), possibly through the reported substance P-induced increase in intracellular calcium (Womack *et al.*, 1988; Murase *et al.*, 1989).

Fig. 1: Comparison of the response provoked in a wide dynamic range neuron by low-threshold (hair) by high-threshold (pinch) mechanical stimulation of the excitatory cutaneous receptive field. Note, following the end of the noxious pinch stimulus, a slow, prolonged depolarization associated with an increase in firing. Even when the intensity of firing during hair stimulation was the same as that during noxious stimulation, no after-excitation was observed. The insets show enlargement of parts of the slower trace above. Note the regular firing during the slow depolarization which appears to be due to the sustained depolarization as opposed to the more erratic firing during the period of continuous hair movement. Recording with $K(CH_3SO_4)$ filled electrode; resting membrane potential = -60 mV.

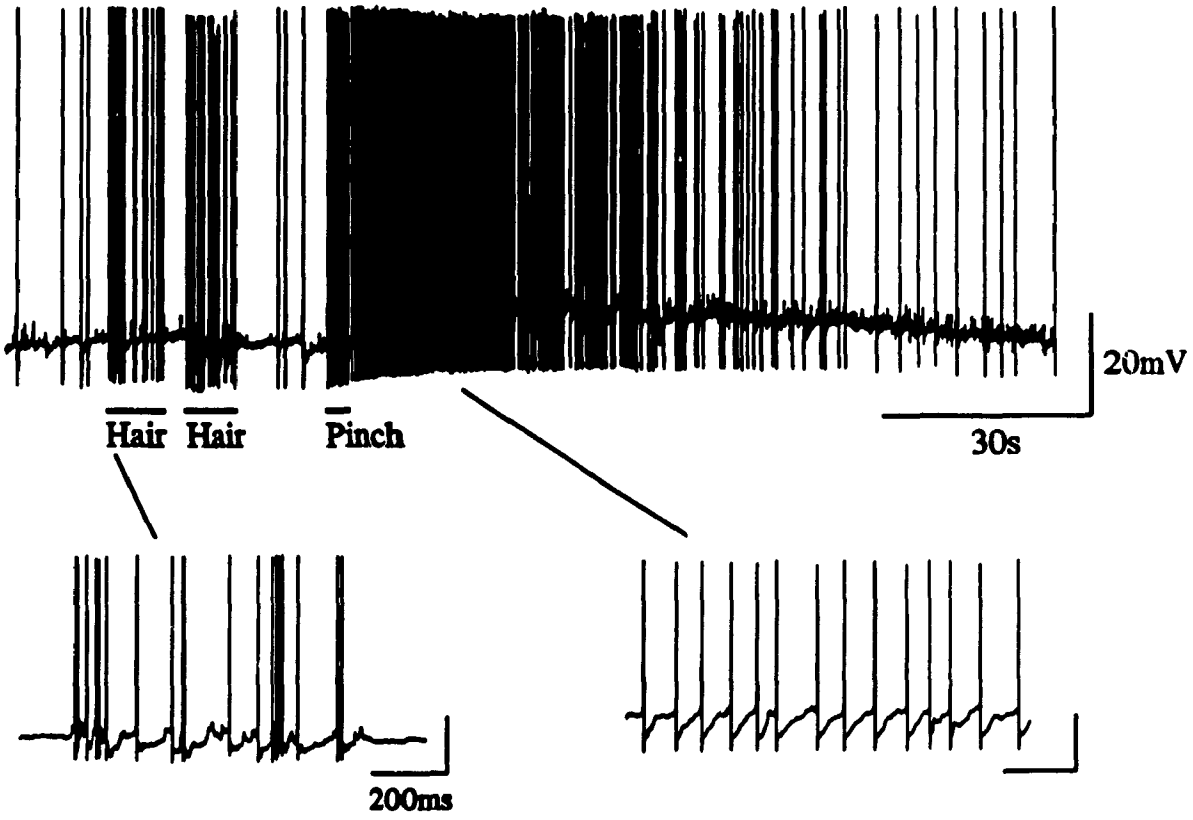

20mV
Hair
Hair
Pinch
30s
200ms

Fig. 2: The prolonged EPSP evoked by a high intensity train of electrical stimuli to the sensory nerve (Tr) is associated with an increase in membrane resistance. The lower record shows the slow depolarization following a train of high intensity electrical stimulation (Tr; 5 mA, 1 ms duration, 15 Hz for 8 s) to the superficial peroneal nerve. Action potentials are filtered out to emphasize the depolarization. The downward deflections are in response to constant current pulses delivered regularly to measure changes in membrane resistance (0.8 nA). Note the decrease in membrane resistance during the train stimulation which contrasts with the increase during the slow prolonged depolarization following the train. The insets at the top —showing enlargements of selected periods before and after the train— show clearly the increase in membrane resistance (larger voltage deflections). A similar depolarization of the membrane potential with continuous intracellular current injection did not cause such an increase in membrane resistance as seen during the slow depolarization showing that the latter increase in resistance is not due to the change in membrane potential *per se* (not shown). Recording with $K(CH_3SO_4)$ filled electrode; resting membrane potential = -62 mV.

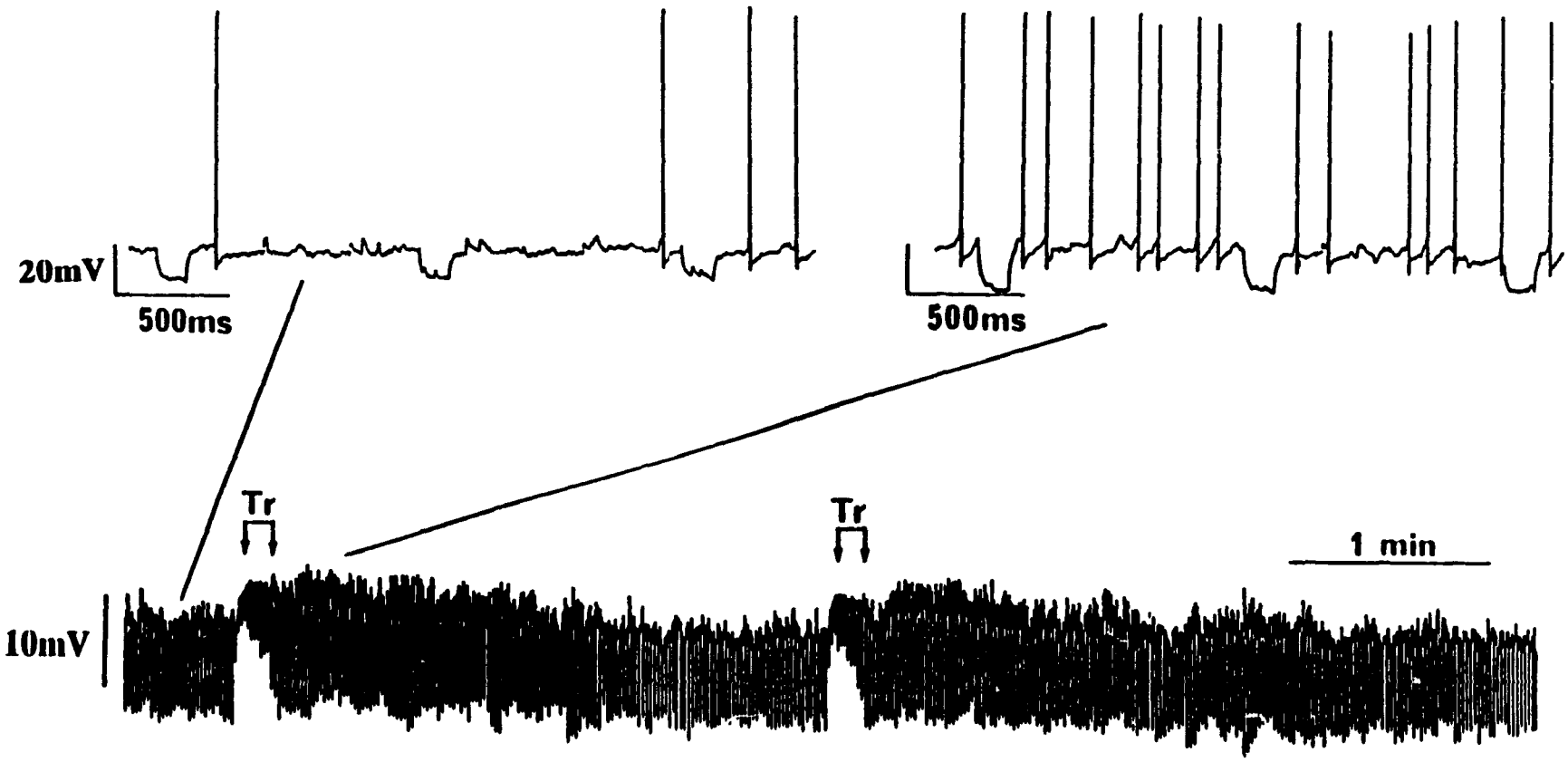

20mV
500ms
500ms
Tr
Tr
1 min
10mV

Fig. 3: Comparison of the reversal potentials of the slow EPSP following a train of high intensity electrical stimuli to the sensory nerve with the late C-fiber-evoked EPSPs in response to a single stimulus. *Left*: Five superimposed traces of responses to single electrical stimuli at C-fiber intensity. The EPSPs seen at C-fiber latency are increased by hyperpolarization and decreased by depolarization suggesting a reversal potential similar to that observed with conventional EPSPs. *Right*: slow prolonged EPSP following a train of high intensity stimuli (10 mA, 1 ms duration, 15 Hz for 10 s) to the superficial peroneal nerve. Note the reversal of the early response during the train stimulation at depolarized potential (top trace); this probably represents a barrage of Cl^--mediated IPSPs overriding the early EPSPs. The late, slow EPSP was increased at depolarized potentials and was abolished at hyperpolarized potentials, as has been seen with substance P-induced depolarization in the *in vitro* spinal cord preparation (Nowak and Macdonald, 1981; Nowak and Macdonald, 1982; Murase *et al.*, 1982; Murase and Randić, 1984). DC current injections from top to bottom: +3.5, +2.5, +1.5, 0 and -2nA. The difference in voltage sensitivity of the late EPSP to a single stimulus versus that of the slow EPSP to a train of stimuli suggests the involvement of different mechanisms. Recording with a KCl filled electrode.

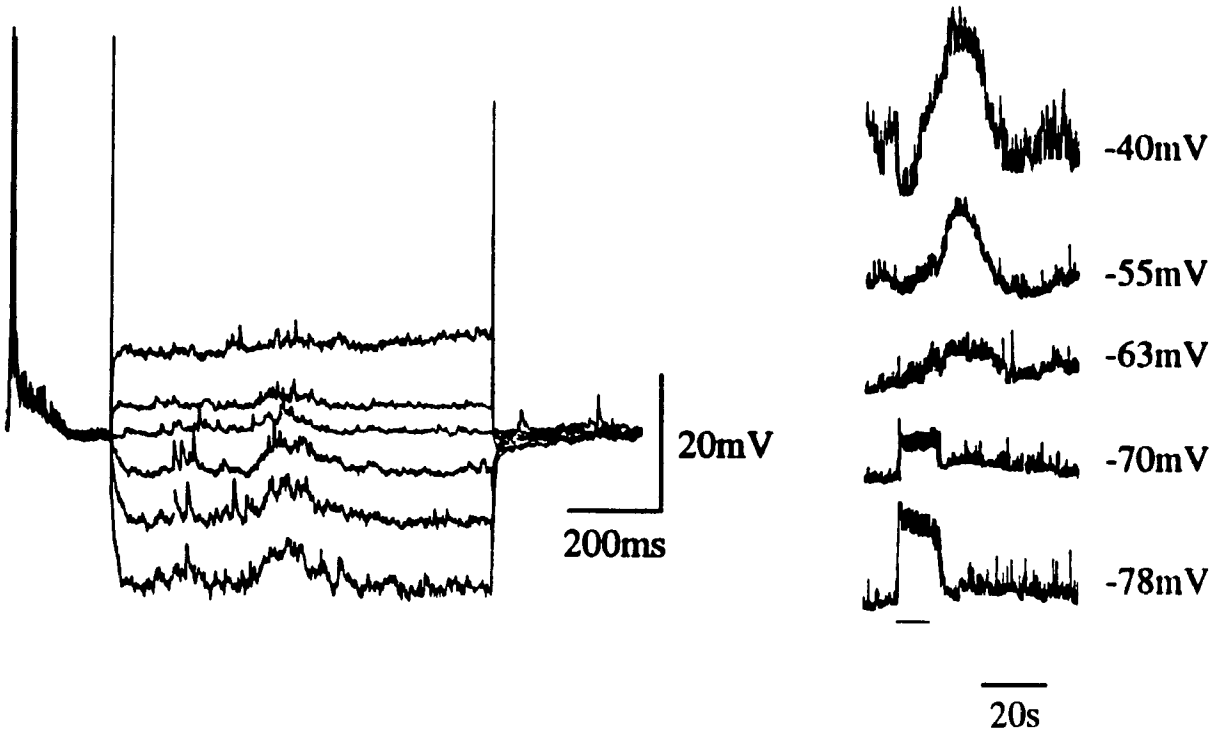
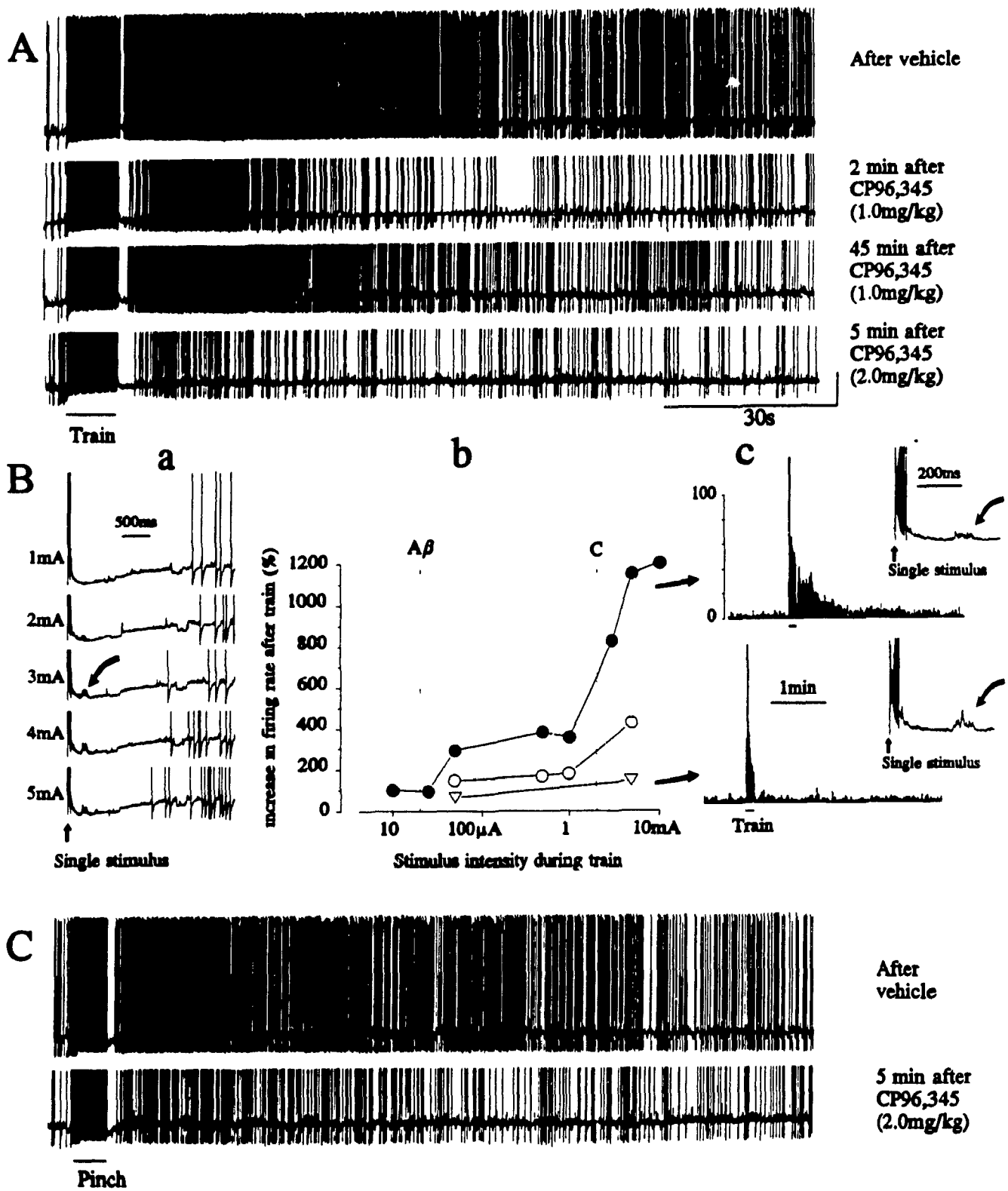

20mV
200ms
-40mV
-55mV
-63mV
-70mV
-78mV
20s

Fig. 4 CP-96,345 reversibly and repeatably blocks the after-excitation following noxious cutaneous pinch and following a train of high intensity electrical stimuli to the sensory nerve. All the data in this figure are taken from the same wide dynamic range dorsal horn neuron. **A:** Four intracellular records showing the response to trains of high intensity electrical stimulation (5 mA, 0.8 ms duration at 12 Hz for 8 s) of the superficial peroneal nerve. Except for the top trace, the action potentials are truncated for clarity of illustration. The top trace is after vehicle injection (four identical preceding trains gave a stable and comparable after-excitation). The second trace was taken 2 min after CP-96,345 was injected (I.V., 1.0 mg/kg). The third trace shows the response 45 min after the second trace and shows some recovery of the slow after-excitation. The bottom trace was taken 5 min after a second dose of CP-96,345 (cumulative: 2.0 mg/kg) and shows further reduction in the response. Note that the response occurring during the train was not depressed (second and the bottom traces; PSTHs on the left of the graph in B) and the resting membrane potential remained unchanged. (Scale bars: vertical = 20 mV, horizontal = 30 s) **B:** Comparison of the response to single electrical stimuli to the sensory nerve with the after-excitation following a train of electrical stimuli to sensory nerve. The traces in **a** show the threshold intensity (2 mA) for the occurrence of the late C-fiber latency EPSP (curved arrow) in response to a single stimulus at increasing intensities. Note that, at intensities above 3 mA, the size of the C-latency EPSP does not further increase. The graph in **b** shows the increase in the firing rate during the after-excitation (60 s window) following the train against intensities of stimulation during the train. ● Records taken before CP-96,345 administration. Note the lack of an after-

excitation at intensities which recruit only Aβ fibers, and the marked increase of the response above the C-fiber threshold. O After the first CP-96,345 injection and Δ after the second CP-96,345 injection. The peri-stimulus time histograms in c represent examples of the spike counts (bin width = 1 s) at 5 mA intensity (during the train) before the first and after the last CP-96,345 administration. Note that the C-fiber evoked EPSPs (curved arrows; insets) were not affected or were perhaps even slightly increased following CP-96,345 administration. C: Two intracellular records showing the responses to noxious pinch stimulation of the excitatory receptive field of the dorsal horn neuron. The spikes are truncated in the lower trace for clarity of illustration. Note the pronounced after-excitation following the end of the pinch stimulus (top trace). Three comparable stimuli gave reproducible responses. The lower trace shows the response to the same cutaneous stimulus 5 min after CP-96,345 administration. Note the marked depression of the after-excitation. However, the response during the pinch was unaffected; the second pinch stimulus was slightly longer and stronger in intensity in an attempt to ensure that the after-excitation was blocked and that the reduction in the after-excitation was not due to a weaker pinch stimulus. Response to hair stimulation was also unchanged (not shown). (Same scale as in A; recording with a $K(CH_3SO_4)$ filled electrode.


A
After vehicle
2 min after CP96,345 (1.0mg/kg)
45 min after CP96,345 (1.0mg/kg)
5 min after CP96,345 (2.0mg/kg)
30s
Train
B
a
500ms
1mA
2mA
3mA
4mA
5mA
Single stimulus
b
Aβ
C
increase in firing rate after train (%)
1200
1000
800
600
400
200
0
10
100μA
1
10mA
Stimulus intensity during train
c
100
0
200ms
Single stimulus
1min
Single stimulus
Train
C
After vehicle
5 min after CP96,345 (2.0mg/kg)
Pinch

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CHAPTER VII

SPINAL DORSAL HORN NEURONES RESPONDING TO NOXIOUS INPUT WITH A SLOW, PROLONGED EPSP ARE CHARACTERIZED BY THE PREFERENTIAL DISTRIBUTION OF APPOSED SUBSTANCE P-CONTAINING VARICOSITIES

ABSTRACT

Substance P has been proposed to mediate a slow, prolonged EPSP in dorsal horn neurones in response to noxious cutaneous stimulation. However a direct anatomical correlation between the presence of such a nociceptive response and substance P input has been lacking. To address this issue, in the present study we combine intracellular recording and labelling of dorsal horn neurones *in vivo* with the immunocytochemical demonstration of substance P at the electron microscopic level using a bispecific monoclonal antibody. A positive correlation was observed between the presence of this slow, prolonged EPSP to noxious cutaneous stimulation and the presence of apposed substance P immunoreactive varicosities onto the same dorsal horn neurone, suggesting that substance P may be the direct mediator of this EPSP in nociceptive dorsal horn neurones.

Substance P has been implicated in the mediation of a period of hyperexcitability in a spinal nociceptive reflex following noxious cutaneous stimulation (Cridland and Henry, 1988). We have shown in chapter VI that, in nociceptive dorsal horn neurones *in vivo*, noxious cutaneous stimulation elicits a slow, prolonged EPSP with a time course similar to that of the period of hyperexcitability. This EPSP was blocked by administration of an NK-1 receptor antagonist. However the question remained whether this slow EPSP is correlated with the incidence of direct synaptic innervation by substance P immunoreactive fibres onto these neurones.

To address this question we recorded intracellularly from dorsal horn neurones *in vivo* in the cat spinal cord and characterized the responses of each neurone to natural stimulation of the cutaneous receptive field and to electrical stimulation of sensory nerves. Each neurone was then injected with horseradish peroxidase (HRP). Following the injection, the tissue was fixed and processed for the demonstration of intracellular HRP and for the presence of substance P-like immunoreactivity using a substance P monoclonal antibody. Thence the relationship between the characterized dorsal horn neurone and the immunoreactive substance P was studied at both the light and electron microscopic levels.

Methods for the preparation of the cats are described elsewhere (De Koninck and Henry, 1989). Briefly, adult cats were anaesthetized with α-chloralose (60 mg/kg i.v.). Recording was at the L_5-L_7 spinal level. The spinal cords were transected at the L_1 vertebral level to eliminate the influence of supraspinal structures. Cats were paralysed

with pancuronium bromide (Pavulon, Organon; 1 mg/kg i.v.) and ventilated artificially. Glass micropipettes were filled with 4-8% HRP (Sigma type VI) in 0.5 M KCl or $K(CH_3SO_4)$ and 0.05 M Tris buffer adjusted at pH 7.5 (resistances: 50 to 120 MΩ). Functional classification of each neurone was done on the basis of the responses to natural cutaneous stimulation and electrical stimulation of the afferent nerves following previously described scheme (Salter and Henry, 1985; Chapter VI). Natural stimuli used were movement of a single hair, noxious pinch with a serrated forceps, innocuous and noxious pressure, noxious radiant heat and vibration using a feedback controlled mechanical stimulator. Neurones were classified as non-nociceptive, wide dynamic range and nociceptive specific. Special emphasis was made to characterize the after-response to noxious stimulation. HRP was injected by intracellular iontophoresis using 600 ms positive current pulses of 2-10 nA at a frequency of 1 Hz for a duration of 10-30 min.

The anaesthetized animal was perfused (1 to 10 hours after the HRP injection) through the left ventricle with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The relevant segments of the spinal cord were sectioned parasagittally at 50 μm in a Vibratome, treated for the demonstration of HRP (Ribeiro-da-Silva *et al.*, 1989) and examined under the light microscope. The intracellularly injected neurone was photographed at this stage. The sections containing the cells were then processed for electron microscopic immunocytochemistry and flat-embedded in epon as previously described (Ribeiro-da-Silva *et al.*, 1989). The antibody

used was an anti-substance P/anti-HRP bi-specific monoclonal antibody from the rat (coded P4C1; Medicorp, Canada) (Suresh *et al.*, 1986). The neurones as observed in flat-embedded slices were drawn with a camera lucida and photographed. After reembedding, 4 μm-thick plastic sections were obtained serially, photographed and compared to the original drawings for the identification of the parts of the intracellularly injected cell present in each section. They were then reembedded and sectioned for electron microscopy. Counts of the number of substance P-immunoreactive varicosities apposed to the dendritic processes and cell body of the intracellularly labelled cells were carried out and the results expressed as a percentage of the total number of boutons apposed onto the membrane.

The present study focuses on the presence of a slow, prolonged EPSP following noxious cutaneous stimulation because this response is blocked by a nonpeptide, substance P receptor (NK-1) antagonist *in vivo* (chapter VI). The excitatory response to noxious mechanical stimulation is different from that to non-noxious mechanical stimulation in that only the former is associated with a prolonged period of after-excitation (in the form of a slow, prolonged EPSP) following the end of the mechanical stimulus (see Fig. 1). Such an after-excitation was not seen with innocuous stimulation, suggesting that the after-excitation is a specific nociceptive response. Hence, neurones were classified on the basis of the presence or absence of this EPSP.

Of the 20 neurones studied, eight are included in this study because they yielded reliable results: their responses to natural stimuli were fully characterized, they had a stable membrane potential and spikes with overshoot and they were successfully labelled intracellularly with HRP.

Four of these neurones responded to noxious stimulation with the slow, prolonged EPSP shown in Fig. 1. Of these cells, one was a nociceptive specific neurone located in lamina I and the remaining three were wide dynamic range neurones located in laminae III-VI. The lamina I neurone was multipolar, with elongated, scarcely branched dendrites which were virtually devoid of spines. Some of the dendritic branches penetrated the white matter of the dorsal columns, while others were directed ventrally reaching as deep as the lamina II-III border. The somata of 2 additional cells were located in lamina V. The cell bodies were ovoid, and gave rise to 3-5 main dendritic trunks. Very few dendritic branches reached lamina VI, most of the dendrites being oriented dorsally. One to two of the dendritic trunks continued as very elongated dendrites reaching as far dorsally as the lamina I-II border. These dendrites were virtually devoid of spines and were seldom postsynaptic to the central elements of synaptic glomeruli. The three neurones described above were apposed by a considerable number of substance P immunoreactive varicosities, both on the cell body and on the dendrites (Fig. 1 and table 1). The fourth cell was stellate in shape and the cell body was located in lamina II. It gave rise to profusely branched dendrites. Two of the dendritic trunks were oriented

dorsally and reached the lamina I-II border. The remainder of the dendritic tree was oriented ventrally and branched extensively in lamina III. Surprisingly, although most of the dendritic branches in lamina III had numerous spines, those in laminae I and IIo were virtually aspiny and these were precisely those where most of the appositions from substance P-immunoreactive varicosities were located. This neurone had a lower number of appositions from substance P-immunoreactive varicosities than the three previous neurones over most of the surface of the cell. All four neurones responded with the slow, prolonged EPSP to noxious stimulation following the end of the noxious stimulus.

With the remaining four neurones, noxious stimuli to the skin and high intensity electrical stimuli did not produce the slow, prolonged EPSP. They showed brisk and brief responses to low-threshold hair input yet only weak, if any, after-excitation to noxious stimulation. In these neurones the weak after-excitation was characterized by an increased rate of discharge and increased, fast synaptic activity. However, this after-excitation was never associated with a slow, prolonged EPSP.

Of these four neurones, two had morphological characteristics similar to spinocervical tract neurones (Brown, 1981): they were multipolar neurones in which the cell body was located in lamina IV, with dendritic trunks directed dorsally. Furthermore, their dendritic tree was elongated rostrocaudally, extended dorsally to lamina II and possessed numerous axon-like spines. Their axon could be traced to the dorso-lateral funiculus. In a third cell, the soma was located in lamina III, the dendrites were oriented

dorsally, with numerous but rather short spines. Its axon projected in the dorsal column, indicating that this cell may belong to the dorsal column postsynaptic pathway. The fourth neurone was located in lamina IIi and its axon arborized locally. The cell was multipolar, with dendrites reaching lamina IIo and lamina III and possessing numerous elongated dendritic spines. With all four neurones, the cell body and most of the dendritic tree were located in regions poor in substance P-immunoreactive varicosities. All had numerous spines (see Fig. 1 for representative example). Furthermore, as illustrated in Fig. 2B, the dendritic processes were frequently postsynaptic to the central varicosities of synaptic glomeruli in laminae II and III (Gobel, 1974; Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva *et al.*, 1985).

As the slow, prolonged EPSP observed with the first group of neurones (Fig. 2) has been shown to be blocked by an NK-1 receptor antagonist (chapter VI), the striking observation here is that neurones associated with this EPSP are also highly associated with substance P-immunoreactive terminals. This contrasts with the lack of substance P-contacts observed with neurones that did not exhibit this slow, prolonged EPSP (table I).

It is interesting to note that the neurones which responded with a slow, prolonged EPSP to noxious pinch stimulation possessed dendrites mainly devoid of spines, the dendrites were not associated with synaptic glomeruli. They were apposed by a relatively large number of substance P-containing varicosities. On the contrary, neurones that had brisk low-threshold hair responses had very spiny dendrites and dendritic processes which

were often postsynaptic to the central varicosities of glomeruli. They were mainly devoid of any type of substance P-immunoreactive input. An intermediate type of synaptic arrangement was observed in one of the wide dynamic range neurone included in this study. This cell did respond with a slow EPSP to noxious stimulation, but this response was not as pronounced as that seen with the other nociceptive neurones. In addition, the neurone had a brisk response to hair stimulation similar to that seen with some of the neurones in the second group (see Fig. 2 for example). The dendritic tree of this neurone was of both types, some parts lacking spines but rich in substance P input, yet other areas rich in spines and frequently associated with synaptic glomeruli but poor in substance P input. These findings suggest a direct association of dendritic spines with hair input and a postsynaptic localization in synaptic glomeruli. They also suggested an association of substance P input with dendritic processes poor in spines and unrelated to glomeruli. The functional significance of this association remains to be elucidated, but we can speculate that it may reflect the difference in time-course of the synaptic responses related to these two inputs.

In conclusion, the association of direct substance P synaptic contacts with neurones exhibiting a slow, prolonged EPSP to noxious input, together with the fact that this EPSP is blocked by an NK-1 receptor antagonist (chapter VI), provides further evidence that substance P is the direct mediator of such an EPSP. The direct action of substance P on the postsynaptic membrane of nociceptive dorsal horn neurones may thus

account for the period of hyperexcitability following noxious input. This period of hyperexcitability may be the neuronal substrate underlying central components of hyperalgesia following noxious input.

Fig. 1. Dorsal horn neurone responding with a slow, prolonged EPSP following noxious cutaneous stimulation. The cell body, located in lamina V (see *camera lucida* reconstruction on the right), was associated with a discrete area of intense Substance P immunoreactive fibres (see Ba).

A: Response of the dorsal horn neurone to natural stimulation of the cutaneous receptive field, which is shown in the schematic diagram: the darkened area represents the low-threshold touch receptive field and the larger hatched area, the high-threshold pinch receptive field.

a: Response of the neurone to low-threshold mechanical stimulation (touch). The period of application of each of 2 stimuli is represented by the horizontal bars below the trace. The inset represents the enlargement, on a faster time scale, of a typical burst of action potentials riding on EPSPs during the stimulus. The calibration for the top trace is the same as in b; the scale bars for the inset represent 20 ms (horizontal) and 50 mV (vertical). The scale bar for the *camera lucida* drawing is 250 μm

b: Response of the neurone to high-threshold mechanical stimulation (pinch). The period of the stimulus is represented by the horizontal bar below the trace. Note the marked depolarization during the noxious stimulus associated with action potential inactivation. Note also the prolonged depolarization following the end of the stimulus associated with an increased firing frequency.

B: Light and electron microscopic analysis of the association between the intracellular filled neurone and substance P immunoreactive profiles.

a: Light microscopic photograph of a parasagittal, 4 μm-thick, epon-flat-embedded section showing part of the cell body of the neurone. Note the presence of numerous substance P immunoreactive profiles apposed to the cell body (*arrows*). The cell body was located within a region of intense immunoreactivity corresponding to a network of substance P-immunoreactive fibres commonly found in lamina V (Ruda *et al.*, 1986). (scale bar = 20 μm).

b: Electron microscopic photographs of an ultrathin section taken from the 4 μm-thick section shown in **a**. Note the two substance P-containing varicosities (*arrows*) apposed onto the cell body. Synapses between these varicosities and the cell body could be demonstrated in an adjacent section (not shown). (scale bar = 1 μm)

c: Electron microscopic photograph of an ultrathin section showing a substance P-containing varicosity (*filled arrow*) in contact with a dendrite of the cell. Note the synapse pointed to by the *open arrow*. The varicosity shown in this micrograph corresponds to the same as that shown on the left in **d** (*arrow* on the left). (scale bar = 1 μm)

d: Electron micrographs of an ultrathin section taken from the 4 μm-thick section in **e**. The portion of the dendrite shown in **d** corresponds to the curved portion of the dendrite shown in **e**. The immunoreactive profiles (*arrows*) belong to an axon which appears to wrap in a spiral-like fashion around the dendrite (*arrows* in **e**) of the cell and make several contacts with the dendrite (scale bar = 1μm). The stars indicate non-immunoreactive varicosities apposed onto the dendrite.
(scale bars in d = 1 μm; in e = 20 μm)

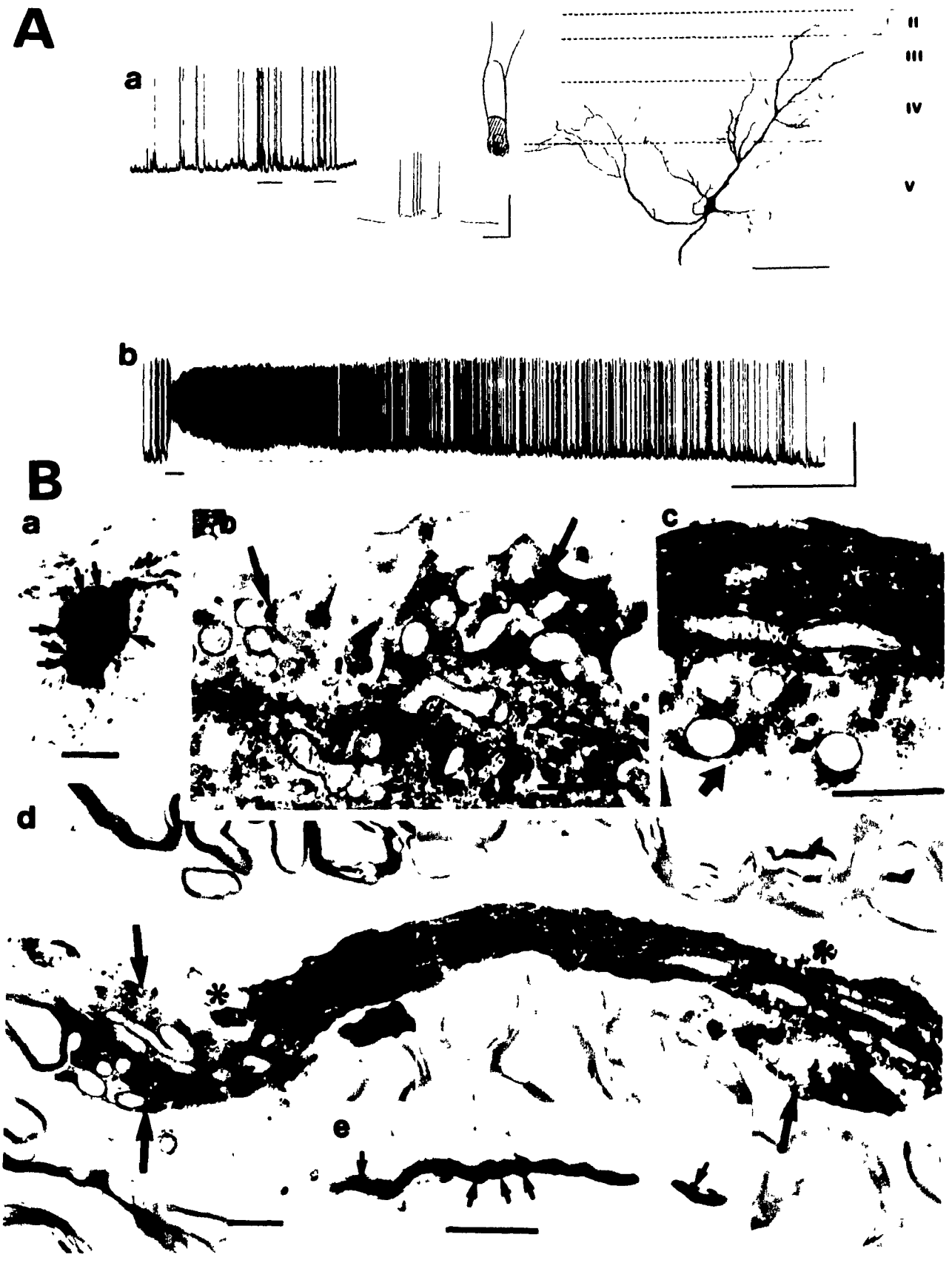
A
a
II
III
IV
V
b
B
a
b
c
d
e

Fig. 2. Lamina III neurone which did not respond with a slow, prolonged EPSP following noxious cutaneous stimulation. Very few substance P immunoreactive varicosities were found in apposition to the cell body and the dendritic tree of this neurone.

A: Response of the dorsal horn neurone to natural stimulation of its cutaneous receptive field. The receptive field of the neurone is shown by the darkened area on the schematic diagram of the hind leg. In the *camera lucida* reconstruction on the right, note the extensive dendritic arborization in the lamina II-III border, a region were abundant hair afferents terminate (Maxwell *et al.*, 1982; Shortland *et al.*, 1989; Réthelyi *et al.*, 1982) (scale bar = 250 μm). Note also the presence axon-like dendritic spines (*inset*), typical of spinocervical neurones (Brown, 1981).

a: Response of the neurone to low-threshold mechanical stimulation (hair). The period of the stimulus is represented by the horizontal bar below the trace. Note the hyperpolarization immediately following the end of the stimulus. The inset represents the enlargement, on a faster time scale, of typical bursts of action potentials riding on EPSPs during the stimulus. Spikes in the upper and lower traces are truncated for clarity of illustration. Scale bars are 3 s (horizontal) and 10 mV (vertical) for the top trace, and 20 ms and 20 mV for the inset.

b: Response of the neurone to high-threshold mechanical stimulation (pinch). The period of the stimulus is represented by the horizontal bar below the trace. Note the on and off response similar to the response to hair stimulation in a. Following the end of

the stimulus, the rate of discharge of the neurone increased slightly, but no prolonged EPSP was observed, in contrast to the response of the cell in Fig. 1.

B: Light and electron microscopic analysis of the association between the intracellularly labelled neurone and substance P-immunoreactive profiles.

The inset on the left shows a light micrograph of a parasagittal, 4 μm-thick, epon-flat-embedded section showing a portion of the cell body and proximal dendrites. Note the lack of substance P immunoreactive profiles in the vicinity of the cell body of this neurone.

The middle panel shows an electron microscopic photograph of an ultrathin section showing a portion of a proximal dendrite of the cell. Only one substance P positive varicosity was found in contact with this dendrite. The stars indicate terminals apposed onto the dendrite. (scale bar = 1 μm)

The dendrites of the neurone were frequently postsynaptic to the central varicosity (C) of synaptic glomeruli (Left panel; scale bar = 1 μm).

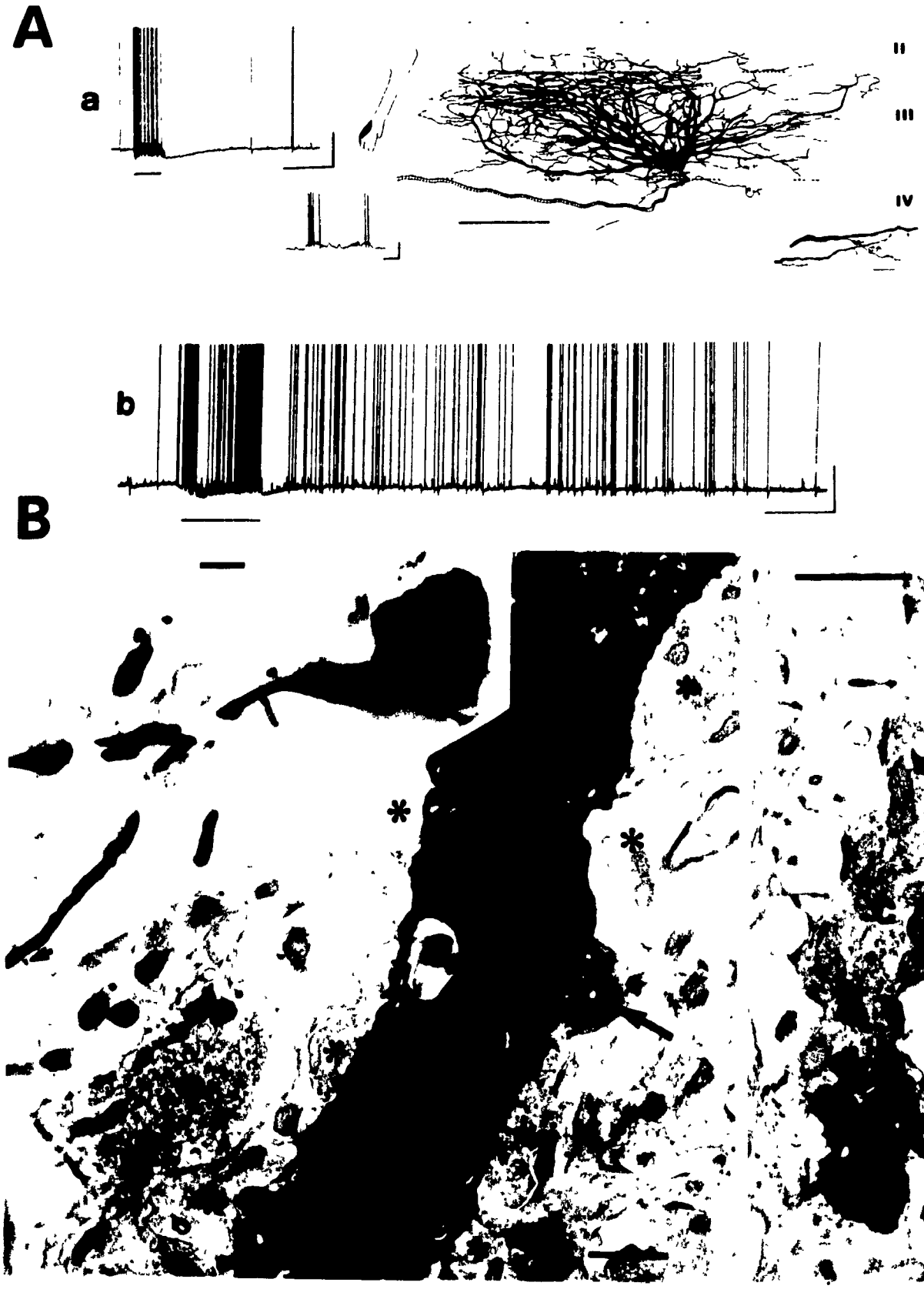
A
a
II
III
IV
b
B

Table I. Neurones associated with a slow EPSP to noxious stimulation possess higher numbers of contacts from substance P-immunoreactive varicosities.[1]

Cell type	Cell body localization	slow EPSP	Substance P-like immunoreactive boutons (%)	
			Cell body	Dendrites
Nociceptive-specific	Lamina I	+++++	39	38
Non-nociceptive	Lamina III-IV	-	2	0
Wide dynamic range	Lamina III	++	7	20[2] 5[3]
Wide dynamic range	Lamina V	++++++	19	29

[1] Expressed as % of total varicosities apposed to the membrane of intracellularly injected neurons; data from one cell per type. Counts of the number of substance P-immunoreactive varicosities apposed to the dendritic processes and cell body of the intracellularly labelled cells were carried out in sections containing different portions of the cell body and dendrites for each neuron. The total number of varicosities in contact with the neuron were counted in each of the sections and the results expressed as a percentage of the total number of boutons: 800 counted per cell.

[2] Portion of dendrites located in lamina I-II

[3] Portion of dendrites located in lamina III

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CHAPTER VIII

GENERAL DISCUSSION

DEVELOPMENT OF A VISUAL SCHEMATIC REPRESENTATION OF THE SYNAPTIC MECHANISMS STUDIED

The results presented in chapters II - VII have provided evidence for the participation of a number of chemicals in specific synaptically elicited responses in dorsal horn neurones. Fig. 1 gives a diagrammatic summary of the different synaptic responses that have been described. The diagram is a simplified and schematic representation and by no means pretends to give a complete or absolute description of the chemical communication mechanisms studied here. For the sake of clarity, all the inputs are illustrated onto the same dorsal horn such as one of the lamina III neurones described in chapter VII which received both hair input and responded to noxious pinch with a prolonged after-excitation (this neurone had a number of spiny dendrites in lamina III and two major non-spiny dendrites extending in lamina I). To simplify the text, we shall refer to this neurone as the L3 neurone.

Excitatory response from hair afferents

Hair afferents are thought to release glutamate which probably acts mainly on non-NMDA receptors in the dorsal horn (chapter V; Hill and Salt, 1982; Jahr and Jessell, 1985). The diagram illustrates a postulated difference in the neuronal circuitry linking a guard-hair afferent and a down-hair afferent to the hypothetical L3 neurone described above.

Both afferents are illustrated in direct contact with the L3 neurone because we have recorded, in the same dorsal horn neurone, responses which appeared to be from both types of afferents and were probably monosynaptic (Chapter V), although this does not exclude other possible linkage as well.

The guard-hair afferent is illustrated with a number of collaterals in contact with a number of hypothetical interneurones to try to account for the apparent large polysynaptic components of the responses from input from a single guard-hair (chapter V; Brown *et al.*, 1987; Hongo and Koike, 1975). On the other hand, the down-hair afferent is represented with a simple connection to the L3 neurone to emphasize the hypothesis that down-hair input triggers a simpler central network than the guard-hair (chapter V; Koerber and Mendell, 1988). This does not exclude the possibility of a more complex network for down-hair afferents. Such a difference in the organization of the two inputs may account for the high *gain* in the central response to a single action potential in a single guard hair afferent ($>$ 1 spike in the L3 neurone per spike in the afferent), contrasting with the low *gain* of the central response to a single down-hair afferent input ($<$ 1 spike in the L3 per spike in the afferent) (situation a).

In the network of feed forward interneurones described above which are associated with the guard-hair input, a number of hypothetical GABAergic inhibitory interneurones are added to account for the marked run down of the central response at increasing frequencies of firing in the afferent (Chapter V; situation b). These GABAergic

interneurones are illustrated making contacts mainly onto terminals or cell bodies that are presynaptic to the L3 neurone to account for the fact no apparent IPSP was seen in dorsal horn neurones that were excited by input from single guard-hair afferents. Nevertheless one inhibitory interneurone is shown in contact with the L3 cell as this interaction remains possible (chapter IV and V). In addition to a possible direct inhibitory connection to the primary afferent, the inhibitory interneurones are shown to inhibit the feed forward interneurones as it is the polysynaptic component which seems to be particularly inhibited at higher frequencies of firing in the afferent (chapter V). Both pre- and postsynaptic inhibitory synapses are drawn as it is possible that either one or both occur (McLaughlin *et al.*, 1975; Barber *et al.*, 1978; Ribeiro-da-Silva and Coimbra, 1980).

Inhibitory responses from low-threshold mechanical afferents

A second unidentified low threshold mechanoreceptive (LTMR; possibly another guard-hair) afferent is illustrated above the guard-hair afferent in Fig. 1. This LTMR is shown contacting another neurone to represent the fact that it probably arises from an area outside the excitatory receptive field of the L3 neurone (chapter IV). This LTMR is shown to synapse, possibly through other interneurones or directly onto a GABAergic interneurone to cause the $GABA_A$-mediated IPSP described in chapter IV. The feed forward excitatory interneurone linking the LTMR afferent to the GABAergic

interneurone is there represent schematically the prolonged time course of the GABAergic IPSP (chapter IV). Other GABAergic interneurones from within the same network or from a neighbouring network are illustrated to account for the presynaptic inhibition of the GABAergic IPSP in the L3 neurone (chapter IV). Hence the IPSP elicited in response to a single mechanical pulse (situation **a**) and the IPSP in response to a train of mechanical pulses (situation **b**) are not markedly different as we have shown that this central response does not reliably follow repetitive input at frequencies above 0.5 Hz (chapter IV). The GABAergic interneurone which are apposed onto the L3 neurone may be also inhibited by other GABAergic interneurones either from within the same network or from a neighbouring network.

Finally, another inhibitory interneurone is shown between the network associated with the guard-hair and that associated with the down-hair because part of the inhibition of the response to down-hair input illustrated in chapter V may be presynaptic. Interestingly, as the conduction velocity of the small diameter down-hair afferent is consid ably lower than that of the other large diameter, low-threshold mechanoreceptive afferents, inhibition arising from the faster afferents (Chapter IV and V) masks input at low frequencies (situation **a**). Both the postsynaptic inhibition of the L3 neurone and the possible presynaptic inhibition of the down-hair afferent by the fast afferents are markedly reduced at higher frequencies of input, releasing inhibition on the down-hair input which becomes a more significant component of the overall excitatory response (situation **b**).

It may be speculated in this context that the guard-hair input serves primarily in the sensation of a large transient, whereas activation of down-hair afferents serves in the sensation of maintained repetitive input.

A vibration sensitive afferent, probably a Pacinian afferent, is also illustrated and postulated to release ATP (Salter and Henry, 1985, 1990) which is hydrolysed into adenosine in the extracellular space to elicit a hyperpolarization of the postsynaptic membrane (Salter and Henry, 1985; Phillis and Wu, 1981; chapters II, III and IV). For the same reasons as presented above for the excitatory response from down-hair afferent input, this Pacinian afferent is illustrated with a simple, direct linkage to the L3 neurone to account for (1) the fact that the response to a simple mechanical pulse (situation **a**) gives rise to a smaller IPSP than that seen with the GABAergic IPSP presented above (chapter II, III and IV), and (2) the fact that the purinergic IPSP follows higher frequencies of firing in the afferent (situation **b**). This does however not exclude the possibility of other linkages. Upon a single mechanical pulse (situation **a**), the GABAergic system gives rise to a large IPSP whereas the purinergic system gives rise to a small IPSP. Upon repetitive mechanical pulses, the GABAergic system does not give rise to a larger or more prolonged IPSP as inhibitory feed-forward neurones are included as part of the network (see above; chapter IV). However, under this condition, the purinergic system gives rise to a prolonged IPSP (situation **b**). As was speculated for the different responses to hair input, whereas the $GABA_A$-mediated response may serve

primarily in situations triggered by a large transient, the purinergic response may serve optimally in situations where a continuous repetitive input arrives. It is also interesting to note that this purinergic IPSP follows higher frequencies of stimulation (Chapter III and IV), a property which matches that of the peripheral receptor which has an optimal frequency/response > 40 (Hunt, 1961).

Please note that the diagram in Fig. 1 attempts to account for the different properties of the central responses studied in this thesis by proposing a representation of the possible underlying neuronal circuitry. However, it is recognized, as discussed in chapters IV and V, that the chemical properties of the synaptic mechanisms involved may account, at least in part, for these dynamic properties. For example, the fact that the purinergic IPSP follows relatively higher frequencies of input may be a property of the adenosine-mediated mechanism not shared by the GABA-mediated mechanism.

Nociceptive afferents

A population of small diameter unmyelinated afferents (and possibly some small diameter myelinated afferents) contains both glutamate and substance P (De Biasi and Rustioni, 1988; Battaglia and Rustioni, 1988). A single action potential in the afferent leads to a glutamate mediate EPSP (situation **a**; chapter VI; Schneider and Perl, 1988; Jahr and Jessell, 1985; Yoshimura and Jessell, 1990). A train of impulses will, however, lead to the additional release of substance P which will produce a prolonged EPSP

outlasting the activity in the afferent (situation b; chapter VI and VII; Urban and Randic, 1984). At this stage, the significance of the other synaptic inputs to the postsynaptic cell may be reset to a different level. EPSPs may be potentiated (Randic *et al.*, 1990), but also the purinergic IPSP may be potentiated (Fig. 2; see discussion below; Salter and Henry, 1987a, 1988).

DISCUSSION

The chemicals represented in of Fig. 1 are suggested to be involved in the responses illustrated on the basis of the results that we have obtained and the information available in the literature. This is not an exclusive list. A wide variety of chemicals are implicated in the mediation and the regulation of synaptic transmission in the dorsal horn, especially in the substantia gelatinosa. Each may play a complex role in the mediation or regulation of fast or slow synaptic events. (Descending pathways have also been omitted in our studies to restrict the analysis to afferent inputs and their regulation by systems intrinsic to the dorsal horn.)

The fact that an NK-1 receptor antagonist blocks the slow, prolonged EPSP described in chapters VI and VII does not exclude the possible participation of other intermediates in that response. In addition, this receptor may be involved in mediating

other synaptic inputs as well. Other peptides may be released upon such a stimulus, some of which may have effects that are not necessarily directly measurable in terms of membrane potential and resistance. Nevertheless, the synaptic events described here are emphasized because they can serve to illustrate how the character of the chemical mechanisms underlying those events may provide their specificity. This is probably best illustrated with the dual response obtained with activation of afferents containing glutamate and substance P, where the peptide is perhaps preferentially released at higher frequencies of activity in the afferent and can modify the properties of the postsynaptic membrane for a sustained period of time. In fact, substance P has been described as having a number of actions, among which is the release of calcium from intracellular pools (Womack *et al.*, 1988) which may in turn trigger a cascade of modifications in the properties of the system. This is perhaps best illustrated by the rather unusual interaction between the action of substance P and the adenosine-mediated inhibition of nociceptive dorsal horn neurones that has been described earlier (Salter and Henry, 1987a, 1988). Substance P, but not glutamate or bradykinin was found to potentiate the inhibition produced by exogenous administration of adenosine. However, the inhibition produced by GABA was unaffected by substance P (Salter and Henry, 1987a). Accordingly, the vibration-induced inhibition of nociceptive dorsal horn neurones, which corresponds to the IPSP described in chapter II and III, is also potentiated by exogenous administration of substance P, consistent with the demonstration that this IPSP is mediated by adenosine

(chapter II and III; Salter and Henry, 1987b, 1988). The logical correlate of these findings would be that the slow EPSP that we have described in chapters VI and VII potentiates the inhibition caused by peripheral vibration. In a preliminary study, we have described such a potentiation (De Koninck, 1989). With 5 of 8 nociceptive neurones, a weak inhibitory response to vibration was potentiated following either noxious cutaneous stimulation or a train of high intensity (above the threshold to recruit C-fibres) electrical stimulation to the afferent nerve. This potentiation was seen at a comparable rate of on-going firing suggesting that it is not due to a mere increase in the firing activity of the dorsal horn neurone. In three cases, the after-excitation following a noxious pinch stimulus was abruptly terminated by the vibratory stimulus. Hence, if the after-excitation described here in nociceptive dorsal horn neurones is linked to some periods of hyperalgesia experienced in humans, this abrupt inhibition of the after-excitation may be a correlate of the same phenomenon observed in humans in which hyperalgesia following a noxious stimulus is abruptly terminated by rubbing the skin (Melzack and Wall, 1982). These results suggest that the response involving the action of substance P which leads to a period of increased excitability also increases the efficacy of other inputs, including an inhibitory system which can cancel the direct excitatory action of substance P itself. The mechanism by which this type of potentiation may occur remains, however, to be determined.

In chapter III, we have shown that the adenosine-mediated IPSP appears to be mediated via the activation of the ATP-sensitive K^+ channel. As other transmitter mechanisms have been shown to interfere with this type of channel (Miller, 1990; Roeper *et al.*, 1991), it may be speculated that the interaction observed between the substance P- and the adenosine-mediated responses may involve regulation of this channel. Such an hypothesis assumes that the interaction occurs at the postsynaptic level. One way to test this possibility would be to study the interaction between the effect of both substance P and adenosine on isolated dorsal horn neurones, as was done with glutamate and substance P (Randic *et al.*, 1990).

The teleological question could be raised regarding is the functional relevance of the presence of two (or more) chemically different inhibitory mechanisms from low-threshold mechanical inputs. Indeed, as was illustrated with the guard-hair *vs* the down-hair inputs, the different dynamic properties of the inputs could be accounted for by two different structural systems without the need of different transmitters. However, as illustrated above with substance P and adenosine, the possible special interaction between two chemical mechanisms lead to characteristics that are not shared by other chemical mechanisms, providing an additional level of specificity.

In the diagram in Fig. 1, all the inputs are illustrated onto the same wide dynamic range neurone for the sake of clarity of illustration because it is conceivable that the same neurone may receive all these inputs; a more restricted pattern may well occur with

certain types of neurones. However, the illustration also serves to emphasize the fact that a number of neurones in the dorsal horn receive convergent inputs from very different types of modalities and some of these convergent neurones (*i.e.* the wide dynamic range neurones) project to supraspinal structures (Willis and Coggeshall, 1978; Willis, 1981). This raises the question of how the specificity of the information can be relayed through such convergent systems to higher structures (see Price, 1986). In this regard, it is possible that, just as the impact of a given afferent input is differentially encoded in the dorsal horn neurone depending on the firing pattern in the afferent, the resulting pattern of firing in the output neurone from the dorsal horn may, in turn, be encoded differentially at higher levels. In this context, the same morphological pathway may thus carry different types of information. The specificity of the response then relies on the encoding which can be a consequence of both the structural as well as the chemical organization in a given pathway.

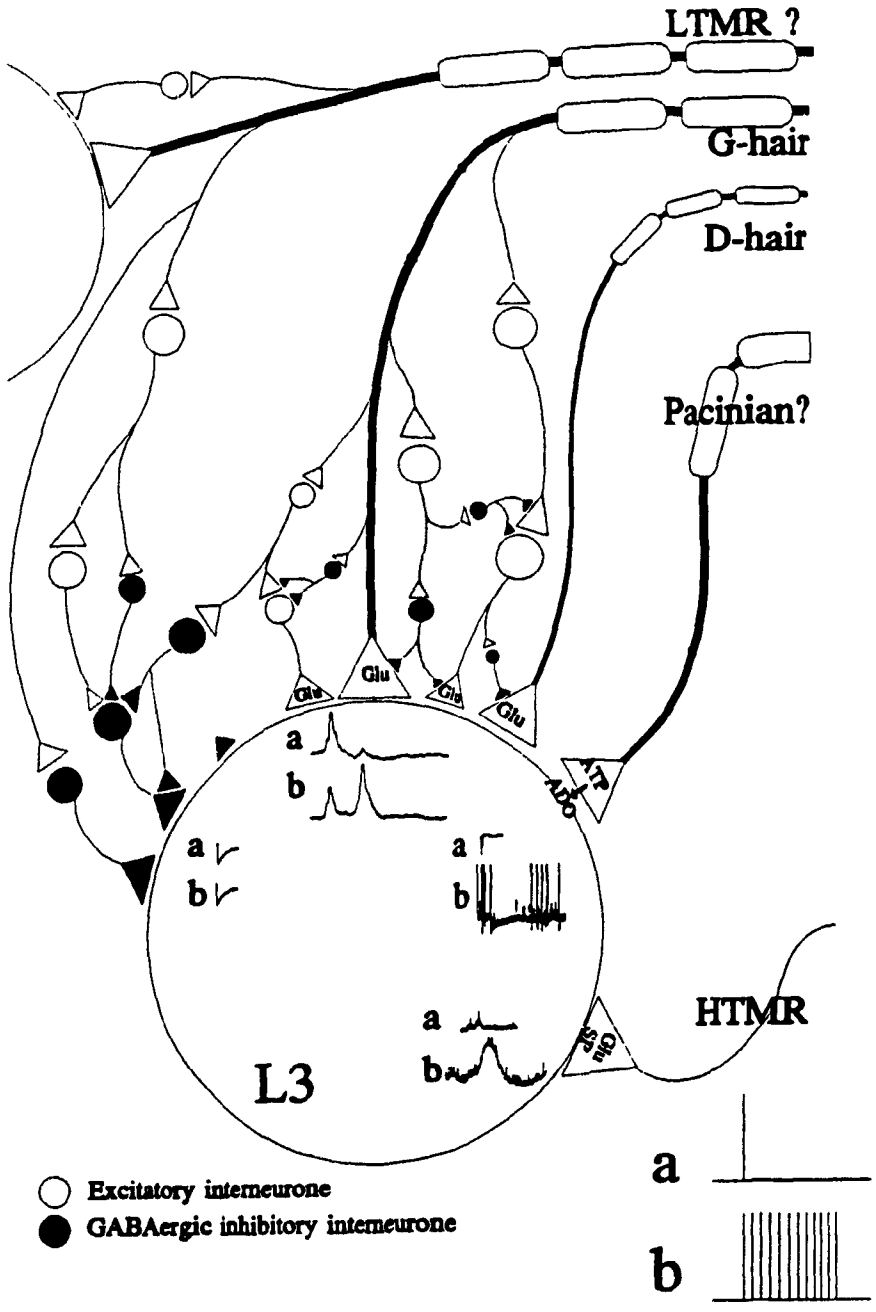

Fig. 1 Schematic visualization of a possible functional organization of the pathways underlying the synaptically elicited responses studied in this thesis. See text for a complete description of the different components of the diagram.

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CLAIMS OF ORIGINALITY

The results and conclusions presented in this thesis are original and have not appeared elsewhere except as specifically mentioned in the text. The principal original contributions arising from the present studies are briefly outlined in the following paragraphs. The specific details appear in the respective chapters.

In chapter II, an adenosine-mediated IPSP was identified in dorsal horn neurones in response to cutaneously applied vibrational stimulation. The identification of adenosine as the chemical mediator of such an IPSP was based on both the similarity between the membrane mechanisms involved in the mediation of this IPSP and the mechanism of action exogenously applied adenosine (*viz.* increase in a K^+ conductance). These results, to our knowledge represent the first demonstration of an adenosine-mediated IPSP in the mammalian central nervous system.

As adenosine had recently been shown to activate ATP-sensitive K^+ channels in cardiac muscle cells, and as the adenosine-mediated IPSP described in chapter II was K^+-mediated, we questioned in chapter III whether ATP-sensitive K^+ channels could be mediating this IPSP. Glibenclamide, a blocker of these channels, blocked the vibration-induced inhibition of dorsal horn neurones. In addition intracellular injection of ATP (to increase the intracellular concentration of ATP to block the ATP-sensitive K^+ channels) was found to block the IPSP evoked by vibration. These results provided evidence of an

IPSP mediated by ATP-sensitive K^+ channels. To our knowledge, this represents the first demonstration of such an IPSP in the mammalian CNS.

As $GABA_A$-mediated inhibitions had also been described in the dorsal horn following activation of low-threshold afferents, we conducted a study in chapter IV, first to confirmed that $GABA_A$-mediated IPSPs occur in dorsal horn neurones and to try to differentiate the GAGA-mechanism from the purine mechanism on a functional basis. It was shown that the $GABA_A$-mediated mechanism responded preferentially to mechanical stimuli at low frequency, which was distinct from the adenosine-mediated mechanism which followed more reliably higher frequencies of stimulation. These results provided a link between a simple adenosine-mediated IPSP to a single pulse and the sustained IPSP observed during a continuous vibration stimulation.

To characterize excitatory inputs to dorsal horn neurones from activation of primary afferents, we conducted three studies to examine responses to activation of a low-threshold mechanical input (hair afferents in chapter V), and high threshold mechanical input (noxious pinch; chapters VI and VII).

In chapter V, we have shown a difference in the character of the response of dorsal horn neurones to guard-hair input and to down-hair input. In addition, we have shown that the prolonged period of inhibition that follows excitatory input from guard-hair afferents involves a $GABA_A$-mediated inhibitory system.

In chapter VI and VII, we provided pharmacological and immunocytochemical evidence that substance P is the chemical mediator of a prolonged period of

hyperexcitability (in the form of a slow, prolonged EPSP) in nociceptive dorsal horn neurones following sustained noxious stimulation. These results thus provided the first evidence of a substance P mediated postsynaptic potential elicited by a natural stimulus *in vivo*.

Finally, the different findings elaborated above were integrated to develop a scheme to account for how both the structural and the chemical properties of the synaptic responses studied in this thesis can yield the functional specificity we know to exist in sensory pathways.