MODULATION OF NEURONAL ACTIVITY IN NUCLEUS RAPHE MAGNUS BY OPIOIDS AND OTHER NEUROTRANSMITTERS IN VITRO

by

Zhi Zhong Pan

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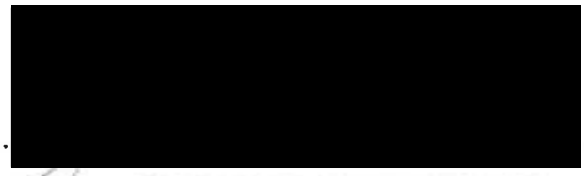

(Professor in charge of Thesis)

(Chairman, Graduate Council)

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ABSTRACT

The nucleus raphe magnus (NRM) has long been implicated in the modulation of nociceptive transmission. There is anatomical, behavioral, pharmacological and physiological evidence suggesting that the midbrain periaquiductal gray (PAG) - the NRM - spinal dorsal horn pathways constitute the major component of the endogenous pain modulating system. Activation of the system, especially the NRM, produces an antinociception. Within the NRM, opioids-, 5-hydroxytryptamine (5-HT)-, acetylcholine (ACh)- and noradrenaline (NA)-containing neurons and/or terminals have been localized and all these compounds have modulating actions on both neuronal activity and spinal nociceptive transmission following local or systemic administration. However, the cellular mechanisms of their actions on the NRM neurons are unknown, nor are the neural mechanisms underlying their modulation on spinal nociceptive transmission. Intracellular recordings were made from neurons in the NRM in slice preparations taken from the rat brain. The cellular actions of opioids, 5-HT, ACh and NA on different types of NRM neurons were studied, including identification of the receptor subtype and the ionic mechanisms. Two types of cells, primary cells and secondary cells, were characterized in the NRM according to their distinct electrophysiological properties. Primary cells had GABA-mediated IPSPs and were predominately 5-HT-containing. Secondary cells displayed only excitatory amino acid (EAA)-mediated EPSPs and did not stain for 5-HT. In primary cells, opioids depressed the GABA IPSP by acting on a presynaptic μ-opioid receptor. This action of opioids provides the physiological evidence for the excitatory effect of opioids through GABA-mediated disinhibition in the CNS and is suggested to be the mechanism underlying opioid activation of the descending inhibitory systems in the NRM in opioid analgesia. Opioids hyperpolarized secondary cells by opening potassium channels through μ-opioid receptors, indicating the roles of these non-serotonergic secondary cells as possible candidates for the intrinsic GABA-containing cells in the NRM. 5-HT, acting on 5-HT_{1A} receptors, hyperpolarized most cells in both primary and secondary cell types by increasing an inwardly rectifying potassium conductance. It is

proposed that the 5-HT-containing primary cells, which could be activated by opioids through disinhibition, may be under control of autoinhibition through 5-HT_{1A} receptors. In a subpopulation of primary cells, muscarinic agonists caused a hyperpolarization by increasing the same inwardly rectifying potassium conductance as that affected by 5-HT. The muscarinic receptor that mediated the hyperpolarization was identified to be of the M_2 subtype. These results suggest an inhibitory postsynaptic cholinergic modulation on the activity of these cells. Present preliminary results demonstrated a complex modulation by NA of primary cells through interactions between an α_1-adrenoceptor-mediated depolarization and an α_2-adrenoceptor-mediated hyperpolarization. NA depolarized secondary cells through α_1 receptors, implying a stronger excitatory influence of noradrenergic inputs on secondary cells.

INTRODUCTION

The nucleus raphe magnus (NRM) is a group of large cells located in the rostral ventromedial medulla. In 1969, Reynolds demonstrated a remarkable phenomenon of stimulation-produced analgesia: local electrical stimulation in the midbrain periaqueductal gray (PAG) produced a strong antinociception that was sufficient to allow a abdominal surgery on an unanesthetized rat. Subsequent mapping studies have revealed that among several sites in the CNS, the NRM is one of the most effective sites for stimulation-produced analgesia (Oliveras et al., 1975). Since then extensive anatomical, behavioral, pharmacological, neurochemical and electrophysiological studies have been carried out to investigate the antinociceptive function of the NRM. For the last decade, the NRM has been receiving growing attention due to the accumulating experimental evidence indicating its primary involvement in modulation of nociceptive transmission at the spinal cord dorsal horn through its direct spinal projections.

Anatomy of the NRM

1. Afferent projections to the NRM

A variety of techniques were used to examine the afferent projections to the NRM, including lesion degeneration, anterogradely or retrogradely transported horseradish peroxidase (HRP) or fluorescence dyes, immunohistochemistry with the combination of transported tracers, etc. It has been demonstrated that the largest afferent input to the NRM originates from the midbrain PAG. An early study showed that following microinjection of HRP into the NRM, two-thirds of the labeled cells were found within the midbrain area (Gallager and Pert, 1978). Later studies using HRP locally injected into the NRM have demonstrated that the most dense afferent projections into the NRM derive from the dorsal and ventrolateral area of the PAG, excluding the ventromedial region known as dorsal

raphe nucleus (Abols and Basbaum, 1981; Beitz, 1982(a); Mantyh and Peschanski, 1982; Marchand and Hagino, 1983; Bockstaele et al., 1991). Other afferent inputs into the NRM were shown to arise from neurons in adjacent medullary reticular formation, nucleus cuneiformis, dorsal and ventral tegmentum, the A5, A7, B8 and B9 cell groups of Dahlstrom and Fuxe (1964), Kolliker-Fuse nuclei and from other structures in cortex, hypothalamus, cerebellum and spinal cord (Abols and Basbaum, 1981; Beitz, 1982a; Maciewicz et al., 1984; Holstege, 1987, 1988).

The neurochemistry of the afferents to the NRM has been examined in previous studies using retrograde and immunocytochemical double labeling techniques. One that has been frequently studied is the PAG-NRM projection. PAG neurons that stain immunocytochemically with antibodies against glutamate and aspartate project to the NRM (Wiklund et al., 1988; Beitz, 1990). In addition, both glutamate and aspartate-immunoreactive terminals were identified in the NRM and chemical stimulation of the PAG by microinjection of homocysteic acid resulted in the release of both glutamate and aspartate in the NRM (Beitz, 1990). A synaptic connection between the PAG terminals and the NRM neurons has been confirmed in an electronmicroscopic study by Lakos and Basbaum (1988). Together with the evidence from electrophysiological and behavioral studies, it is now believed that excitatory amino acids function as a neurotransmitter in the PAG-NRM pathway which is a main component in the endogenous pain modulation networks (Fields et al., 1991).

Anatomical and physiological evidence suggests that other neurotransmitters including neurotensin and 5-hydroxytryptamine (5-HT) are present in the projections from the PAG to the NRM. Neurotensin-containing neurons in the PAG has been identified as a component of the projection to the NRM (Beitz, 1982a; Beitz et al., 1983); Neurotensin-binding sites are localized in the NRM (Quirion et al., 1982; Young and Kuhar, 1981); Neurotensin microinjected into the NRM causes antinociception, indicating a functional role of neurotensin in modulatory influence of the PAG on the NRM (Fang et al., 1987). 5-HT

input from the PAG to the NRM has also been demonstrated (Beitz, 1982a), but its functional significance is still unknown. The major serotonergic input to the NRM has been shown to arise from the midbrain B8 and B9 cell groups of Dahlstrom and Fuxe (1964) (Beitz, 1982a; Beitz et al., 1983).

Noradrenergic inputs have been shown to originate from regions that include the A5 and A7 catecholamine cell groups in the pons and the A1 in medulla (Takagi et al., 1981). A cholinergic afferent projection from the pedunculopontine tegmental nucleus to the NRM is demonstrated by Rye et al (1988). Both of the noradrenergic and the cholinergic inputs have been implicated in the antinociceptive function of the NRM (Proudfit, 1988; Iwamoto, 1991).

2. Efferent projections from the NRM

The ascending and descending efferent projections from the NRM are to many areas in the brain, particularly to structures in the brainstem. Terminals were found in parabrachial nucleus, dorsal raphe, locus coeruleus, prepositus hypoglossi, nucleus tractus solitarius, spinal trigeminal nucleus and the cerebellum (Bobillier et al., 1976; Basbaum et al., 1978; Takagi et al., 1981). The major descending projection which has been extensively studied is from the NRM to the spinal cord.

Efferent projections from the NRM terminate in both dorsal and ventral horns at all levels of the spinal cord (Watkins et al., 1980; Holstege and Kuypers, 1982; Carlton et al., 1985; Skagerberg and Bjorklund, 1985; Basbaum et al., 1986; Mason and Fields, 1989) and descend bilaterally in the dorsolateral funiculi (Holstege and Kuypers, 1982; Basbaum et al., 1986; Jones and Gebhart, 1987). Dense raphe-spinal terminations were found in the marginal zone, the substantia gelatinosa and laminae V, VI and VII of the spinal dorsal horn (Ruda et al., 1981; Holstege and Kuypers, 1982; Basbaum et al., 1986; Jones and Light,

1990). A study by Light and Kavookjian (1985) on the ultrastructure and synaptic connections of the spinal terminations from axons descending in the dorsolateral funiculi from the NRM showed two patterns of raphe-spinal terminations: axons terminating dorsally in laminae I, II, V and X, and axons terminating ventrally in laminae VI, VII and X. Heterogeneity in the terminal ultrastructure was also observed, indicating heterogeneous function of these raphe-spinal projection neurons. Thus the major descending projection from the NRM is identified to terminate in the spinal region where there are dense terminals of nociceptive primary afferents (Light and Perl, 1979; Cervero and Iggo, 1980) and nociceptive neurons that project to the thalamus (Dubner and Bennet, 1983), that is, in the spinal region where nociceptive transmission is believed to occur (Fields and Basbaum, 1978; Basbaum and Fields, 1984). Taken together, the PAG-NRM-spinal dorsal horn pathways constitute a major anatomical component of the endogenous pain modulating system (Basbaum and Fields, 1984).

3. Neurochemistry of the neurons within the NRM

The NRM and the adjacent reticular formation correspond in location to the serotonergic B3 cell group originally described by Dahlstrom and Fuxe (1964). Many neurons in the NRM were identified to be serotonergic by histofluorescence and immunocytochemical studies (Bowker et al., 1981; Steinbusch and Nieuwenhuys, 1983; Wiklund et al., 1981). Anatomical evidence has resulted in the current recognition that the NRM comprises a heterogeneous population of cells. Wiklund et al (1981) reported that only approximately 15% of the total cell population within the NRM was identified to be 5-HT-containing. Cholinergic (acetylcholinesterase-positive) (Satoh et al., 1983), γ-amino-butyric acid (GABA)-containing (Mugnaini and Oertel, 1985; Nagai et al., 1985), enkephalin-immunoreactive (Finley et al., 1981a; Khachaturian et al., 1983; Williams and Dockray, 1983; Menetrey and Basbaum, 1987) and substance P (SP)-containing (Johansson et al., 1981; Bowker et al., 1982; Hancock, 1984) cell bodies and terminals have all been

identified immunohistochemically in the NRM. In addition, dynorphin (Khachaturian et al., 1982), cholecystokinin (CCK) (Kubota et al., 1983; Mantyh and Hunt, 1984), neurotensin (Jennes et al., 1982), somatostatin (Finley et al., 1981b) and thyrotropin-releasing hormone (TRH) (Jahansson et al., 1981) immunoreactivity have also been observed in neurons in the NRM. Adding to the complexity of the NRM neurochemical composition, there is evidence demonstrating the co-existence of 5-HT with either SP or TRH (Jahansson et al., 1981; Bowker et al., 1982), 5-HT with GABA (Millhorn et al., 1987a, 1988) and enkephalin with somatostatin (Millhorn et al., 1987b) in NRM neurons.

A variety of neurotransmitters have been identified as well in the NRM neurons that project to the spinal cord. Many raphe-spinal projection neurons in the NRM contain 5-HT (Bowker et al., 1983; Lovick and Robbinson, 1983; Skagerberg and Bjorklund, 1985). Using retrogradely transported HRP in combination with immunocytochemistry, Bowker et al. (1982) showed that of all the spinal projection cells in medullary raphe nuclei including the NRM, 80% were immunoreactive for 5-HT, 50% stained positively for SP, 10% had TRH-immunoreactivity and 15% showed enkephalin-like immunoreactivity. Co-localization of 5-HT with SP in raphe-spinal projection neurons has also been demonstrated (Hokfelt et al., 1978). Utilizing SP immunocytochemistry in combination with autoradiography following uptake and retrograde transport of [^{3}H]5-HT, Magoul et al (1988) reported that in medullary raphe nuclei, 48% of the 5-HT radiolabeled neurons that projected to the spinal cord stained positively for SP. Moreover, localization of GABA (glutamic acid decarboxylase immunoreactivity) has been confirmed in the spinally projecting neurons in the NRM (Millhorn et al., 1987a; Jones et al., 1991).

Nociceptive modulation by the NRM

1. Stimulation-produced antinociception in the NRM

As mentioned previously, focal electrical stimulation in the NRM produces antinociception (Besson and Chaouch, 1987). Spinal nociceptive neurons which were activated by noxious stimuli were inhibited by stimulation in the NRM (Giesler et al., 1981; Willis, 1982; Gebhart, 1986). Light et al (1986) demonstrated that the noxious stimulus-evoked activity of the laminae I and II neurons in the spinal cord dorsal horn was inhibited by single pulses of stimulation in the NRM. In addition, an inhibitory postsynaptic potential (IPSP) was recorded intracellularly in most nociceptive neurons following the stimulation, suggesting that descending inhibition of the spinal nociceptive transmission from the NRM is a postsynaptic effect.

The NRM also functions as a relay structure in the antinociception mediated through the midbrain PAG (Basbaum and Fields, 1984). Stimulation in the PAG activated neurons in the NRM and caused antinociception (Behbehani et al., 1981; Mason et al., 1985). The inhibition of spinal dorsal horn neuronal responses to noxious stimulation elicited by electrical activation of the PAG was blocked by lesions of or local injection of anesthetics into the NRM (Gebhart et al., 1983; Prieto et al., 1983). Blockade of NRM function or its descending projections in dorsal lateral funiculi significantly attenuated the antinociception induced by systemic administration of opioids or microinjection of opioids into the PAG (Fields and Basbaum, 1978; Basbaum and Fields, 1984).

Thus, there is anatomical, physiological and behavioral evidence supporting the notion that the PAG-NRM-spinal dorsal horn pathways comprise a major component of the endogenous pain modulation network. Activation of descending inhibition from the NRM results in antinociception.

2. Opioids and the NRM

Opioids and antinociception through the NRM It has been well established using a variety of tests that microinjection of opioids into the NRM causes an antinociception (Levy and Proudfit, 1979; Azami et al., 1982; Jensen and Yaksh, 1986, 1989). μ-opioid binding sites in the NRM have been identified by autoradiographic studies (Bowker and Dilts, 1988; Mansour et al., 1988). Microinjection of enkephalinase inhibitors into the NRM produced an antinociception that was reversed by opioid the receptor antagonist naloxone (Al-Rodhan et al., 1990). This implies an involvement of endogenous opioids within the NRM in nociceptive modulation. In addition, the responses of neurons in the dorsal horn to noxious stimuli were inhibited by opioids microinjected into the NRM, but no inhibition was elicited in spinally transected animals (Clark et al., 1983; Du et al., 1984). A similar inhibition of responses of dorsal horn neurons to noxious stimuli was obtained following microinjection of both opioids and glutamate into the NRM (Aimone and Gebhart, 1986; Jones and Gebhart, 1988).

Together with the experiments on stimulation-produced antinociception discussed previously, these results are consistent with the hypothesis that opioids produce analgesia, at least in part, by acting supraspinally to activate descending inhibitory systems in the NRM (Fields et al., 1988). Other supporting evidence for this hypothesis includes the observation that analgesic actions of systemically applied opioids were significantly attenuated by lesions of the spinal dorsolateral funiculi where the inhibitory projections of the NRM descend (Barton et al., 1980). Moreoner, naloxone locally injected into the NRM inhibited the antinociceptive effect of systemically administered opioids (Azami et al., 1982). Although a direct analgesic action of opioids at the spinal cord level has also been confirmed (Yaksh, 1981), Yeung and Rudy (1980) have demonstrated that to elicit a given analgesic effect, concurrent injections of opioids at spinal and supraspinal sites require a much lower total dose than a single large injection at either site. Thus it appears that supraspinal and spinal actions of opioids act synergically.

Opioids and neuronal activity in the NRM If opioids exert analgesic effect, at least partly, by activating the descending inhibitory systems from the NRM, one would expect that opioids should excite the activity of NRM cells, particularly those raphe-spinal projection cells. Actions of opioids on the neuronal activity in the NRM have been examined by systemic or local application of opioids into the PAG. Indeed, many neurons in the NRM were excited by opioids; but it was also found in most studies that a comparable number of NRM neurons were inhibited by opioids and others were not affected (Deakin et al., 1977; Toda, 1982; Gebhart, 1982; Duggan and North, 1983). Chiang and Pan (1985) demonstrated three different responses to systemic analgesic opioids on physiologically identified raphe-spinal projection neurons: one group of cells were excited, the second group of cells were inhibited and the third group of cells were not affected. These results indicate that there are functionally heterogeneous groups of neurons in the NRM as well as the neurochemical heterogeneity discussed before. These results also imply that since there are large number of raphe-spinal projection cells that are inhibited by opioids, the output influence from the NRM onto the spinal nociceptive transmission may not be pure inhibitory, although it is likely dominant as indicated by the previously discussed findings that non-selective electrical and chemical activation of the NRM cause antinociception. It is hypothesized that those raphe-spinal neurons in the NRM which are excited by opioids are probably responsible for the descending inhibition. The function of the raphe-spinal neurons that are inhibited by opioids is less clear.

Opioids and Off- / On-cells in the NRM Fields and colleagues have correlated the activity of cells in the rostral ventromedial medulla (RVM) including the NRM with a functional nociceptive tail-flick reflex in lightly anesthetized rats. Three types of cells were identified in the RVM based on the temporal correlation of the change in spontaneous firing with the occurrence of the tail-flick reflex elicited by a noxious stimulation (Fields et al., 1983, 1988). (1)Off-cells: cells that show a abrupt pause in firing just prior to the tail-flick. (2)On-cells: cells that exhibit a sudden increase in firing just before the tail-flick. (3)Neutral cells: cells that display no change in firing related to the tail-flick. A portion of

cells in each group project to the spinal cord via dorsolateral funiculi (Vanegas et al., 1984). Systemic or local administration of opioids into the PAG, which inhibited the tail-flick, eliminated the pause in Off-cell firing by increasing the spontaneous activity (Fields et al., 1983; Cheng et al., 1986), but decreased the firing of On-cells (Barbaro et al., 1986). Therefore it is proposed that Off-cells exert a net inhibitory effect on spinal nociceptive transmission and a pause in their firing allows the tail-flick to occur; On-cells are hypothesized to have a permissive or facilitating action on nociceptive transmission and their activity facilitates the tail-flick reflex (Fields et al., 1991). This function of On-cells is further supported by a recent observation that inactivation of the NRM by local application of lidocaine attenuated the hyperalgesia (increased pain sensitivity) induced by naloxone-precipitated opioid abstinence. This implies that the CNS including the NRM is capable of generating a facilitating action on nociceptive transmission (Kaplan and Fields, 1991).

Additional evidence indicating the modulatory actions of both Off- and On-cells on nociceptive transmission comes from the findings that the spontaneous firing of the Off- and On-cells was reciprocal in phase in lightly anesthetized rats (Barbaro et al., 1989). The tail-flick threshold was significantly higher during periods of Off-cell (presumably descending inhibitory) activity/On-cell silence and lower during Off-cell silence/On-cell firing (Heinricher et al., 1989).

3. 5-HT and the NRM

5-HT and nociceptive modulation in the NRM The presence of a large number of 5-HT-containing cells, especially 5-HT-containing raphe-spinal projection cells, in the NRM implicates a substantial role of 5-HT in modulation of nociceptive transmission through the NRM. Previous behavioral and pharmacological studies have provided ample evidence for this function of 5-HT: Microinjection of opioids or stimulation in the NRM causes the release (Hammond et al., 1985) or increase in turnover (Vasko et al., 1984) of 5-HT in the

spinal cord; Systemically applied opioids increase the concentration of 5-HT metabolites in the NRM (Rivot et al., 1988, 1989); The antinociceptive effects of stimulation in the NRM and of opioids microinjected into the NRM are antagonized by 5-HT receptor antagonists administered intrathecally at the spinal cord (Hammond and Yaksh, 1984; Jensen and Yaksh,1986). Thus it is believed that 5-HT plays a role in the modulatory function of the NRM on nociceptive transmission.

Unlike the observations of opioid actions, contradictory findings have been reported when investigators have tried to determine the role of 5-HT in the NRM. Local injection of 5-HT or 5-HT receptor agonists into the NRM has been found to cause either analgesia (Llewelyn et al., 1983; Inase et al., 1987), hyperalgesia (Berge et al., 1989; Millan et al., 1989) or no effect (Aimone and Gebhart, 1988). The reasons for the discrepancy are unclear. Consideration of the dose applied, the species studied, the type of nociceptive tests used, and more importantly, multiple 5-HT receptor subtypes and functionally heterogeneous groups of cells in the NRM may explain the discrepancies.

5-HT and neuronal activity in the NRM Like the action of opioids, exogenous 5-HT has been demonstrated to have different effects on the spontaneous activity of neurons in the NRM. In an early study in monkeys, iontophoretic 5-HT inhibited the spontaneous firing in all of seven physiologically identified raphe-spinal neurons that responded (Wilcockson et al., 1983). Subsequent studies in rats demonstrated that the majority of the NRM cells were inhibited by iontophoretic application of 5-HT, although a smaller proportion of cells were excited and a much smaller number of cells were not affected (Wessendorf and Anderson, 1983; Davies et al., 1988a, 1988b; Hentall et al., 1993). These studies confirm the functional heterogeneity of the NRM neurons, but the roles of 5-HT and serotonergic neurons in the NRM in nociceptive transmission remain unclear.

4. Acetylcholine and the NRM

Acetylcholine and nociceptive modulation in the NRM The CNS cholinergic system (both nicotinic and muscarinic) has long been implicated in endogenous modulation of pain transmission (for review see Green and Kitchen 1986). Antinociception is elicited following systemic or local application of cholionmimetics into several CNS sites including the PAG and the NRM (Llewelyn et al., 1981; Brodie and Proudfit, 1984). Compared to studies on opioids and 5-HT, considerably less has been done regarding the function of acetylcholine (ACh) in the NRM-mediated antinociception. Brodie and Proudfit (1986) reported that the antinociception induced by microinjection of carbachol, a muscarinic receptor agonist, into the NRM was antagonized by spinally applied adrenoceptor antagonists, but not by 5-HT receptor antagonists. These results indicate that the carbachol-induced antinociception may not be mediated by the raphe-spinal serotonergic neurons in the NRM. On the other hand, a recent study has demonstrated that local administration of methoctramine, an M_2 muscarinic receptor antagonist, into the NRM produces a strong antinociceptive effect (Iwamoto, 1991). This result suggests that the activity of the NRM output neurons may be under a tonic inhibitory cholinergic control.

ACh and neuronal activity in the NRM As indicated by the behavioral studies, there have been conflicting reports regarding the actions of iontophoretically applied ACh on the spontaneous activity of the NRM neurons. In earlier studies, ACh applied iontophoretically excited approximately 75% of the cells in the NRM (Behbehani, 1982; Wilcockson et al., 1983). Later studies have shown that 64 to 70% of the NRM neurons are inhibited by iontophoretic ACh (Wessendorf and Anderson, 1983; Hentall et al., 1993). The reasons for the discrepancy are unknown at present.

5. Noradrenaline and the NRM

Noradrenaline and nociceptive modulation in the NRM Several lines of evidence suggests that noradrenaline (NA) is involved in the modulation by the NRM of spinal nociceptive transmission. Electrical or chemical stimulation of the NRM neurons resulted in an increase in the release of endogenous NA into spinal cord superfusates (Hammond et al., 1985) and produced antinociception that was blocked by intrathecal injection of noradrenergic antagonists (Hammond and Yaksh, 1984). Although it seems that there are no NA-containing cell bodies present within the NRM, noradrenergic fibers and terminals have been identified (Takagi et al., 1981) and both α_1- and α_2-adrenergic binding sites have been demonstrated in the NRM (Young and Kuhar, 1980; Unnerstall et al., 1984). Microinjection of adrenoceptor antagonists such as phentolamine into the NRM has an antinociceptive effect (Sagen and Proudfit, 1981; Sagen et al., 1983), indicating that there may be a tonic noradrenergic modulation on the activity of the NRM neurons. The source of the noradrenergic terminals in the NRM is believed to derive, at least in part, from the A1, A5 and A7 catecholamine nuclei, the regions that have been implicated in nociceptive modulation (Proudfit, 1988).

NA and neuronal activity in the NRM Although there is not much information available concerning NA action on the activity of the NRM neurons, in a few electrophysiological studies in vivo, the effect of iontophoretic NA were shown to be predominantly inhibitory (Wessendorf and Anderson, 1983; Wilcockson et al., 1983). There is electrophysiological and behavioral evidence indicating that both α_1- and α_2-adrenergic receptors in the NRM are involved in the mediation of both the cellular activity and nociceptive transmission (Fields et al., 1991).

Mechanisms underlying the actions of opioids and these neurotransmitters in the CNS

A key issue to clarifying the neural mechanisms of opioid analgesia is to understand the cellular mechanisms underlying the actions of opioids and these neurotransmitters on neurons. These actions have been extensively characterized throughout the brain.

1. Opioids

The cellular actions of opioids have been well studied in the CNS (see Duggan and North, 1983 for review). The dominant action of opioids on the CNS neurons is inhibition, resulting in both slowing in cell firing and a reduction in transmitter release. The ionic mechanism underlying the inhibitory action of opioids was first demonstrated by Williams et al (1982) in locus coeruleus where opioids hyperpolarize neurons by opening potassium channels. This action of opioids has been elucidated in many regions of the CNS (Duggan and North, 1983). It is now clear that the primary signal transduction mechanism in opioid action is mediated through pertussis toxin-sensitive G_i proteins that can 1) activate a potassium conductance (North et al., 1987); 2) decrease calcium entry through calcium channels (Schroeder et al., 1991); and 3) inhibit adenylate cyclase activity (Loh et al., 1990), although a stimulation of adenylate cyclase activity by opioid receptor agonists has been reported in rat olfactory bulb (Onali and Olianas, 1991). It has been suggested that most of the excitatory actions of opioids in the CNS result indirectly from a disinhibition, as opioids inhibit the activity of inhibitory interneurons in hippocampus (Madison and Nicoll, 1988). A direct excitatory action of opioids has been shown in dorsal-root ganglion cells in culture, where low concentrations of opioid agonists prolong the duration of action potentials which could presumably enhance transmitter release (Crain and Shen, 1990).

At least three distinct opioid receptor types (referred to as μ, δ and κ) exist in the CNS (North, 1986; Mansour et al., 1988). Both μ- (Williams and North, 1984; Wimpey and

Chavkin, 1991) and δ- (Mihara and North, 1986) opioid receptors have been shown to be coupled to an increase in potassium conductance through G-proteins (North et al., 1987). μ- (Schroeder et al., 1991), δ- (Hescheler et al., 1987; Surprenant et al., 1990) and κ- (Cherubini and North, 1985; MacDonald and Werz, 1986; Gross et al., 1987) opioid receptors all act to inhibit voltage-dependent calcium channels.

2. 5-hydroxytryptamine

Recent molecular biological studies have discovered a variety of 5-HT receptors present in the CNS. The development of selective ligands has resulted in the current classification of 5-HT receptor subtypes into three categories: 5-HT_1, 5-HT_2 and 5-HT_3. 5-HT_1 receptors have been further divided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} (Bradley et al., 1986; Peroutka, 1988). Biochemical, physiological and pharmacological studies have demonstrated the mechanism of cellular actions by each of these subtypes and the signal transduction systems with which each subtype is associated (see Bobker and Williams, 1990; Zifa and Fillion, 1992 for reviews). 5-HT_{1A} receptors are coupled to an increase in potassium conductance and associated with an inhibition of adenylate cyclase activity. 5-HT_{1B} receptors found in rat and mouse brain are negatively coupled to adenylate cyclase and presynaptically inhibit the release of 5-HT and other neurotransmitters such as ACh, NA and glutamate. 5-HT_{1C} receptors, similar to 5-HT_2 receptors in pharmacological characteristics, stimulate phosphatidylinositol (PI) turnover and mediate an increase in chloride conductance via inositol 1,4,5-triphosphate (IP_3) and intracellular calcium. 5-HT_{1D} receptors, found in guinea-pig, calf and human where 5-HT_{1B} sites are absent, are similar to 5-HT_{1B} in that both inhibit adenylate cyclase activity and cause presynaptic inhibition of neurotransmitter release. 5-HT_2 receptors that appear to be coupled to PI turnover mediate an excitation of neurons through a decrease in potassium conductance and are involved in control of transmitter release as well. All subtypes described above act

through G-proteins. 5-HT_3 receptors are a receptor-channel complex and activation causes an increase in a non-selective cation conductance.

3. Acetylcholine

While nicotinic cholinergic receptors are a receptor-channel complex mediating excitation through opening of a cation channel, muscarinic cholinergic receptors comprise a G-protein-regulated receptor family of five distinct subtypes, as discussed in the following manuscript. In the CNS, pharmacologically defined M_1 receptors mediate the depolarization of neurons in brain (McCormick and Prince, 1985; Muller and Misgeld, 1986) and presynaptic inhibition of GABA release (Sugita et al., 1991). Previously defined M_2 receptors are reportedly involved in either depolarization (Egan and North, 1985; McCormick and Prince, 1985), hyperpolarization (Egan and North, 1986; McCormick and Prince, 1986) of brain neurons or blockade of afterhyperpolarization (Muller and Misgeld, 1986; Constanti and Sim, 1987). But these results should be reconsidered, as receptors previously defined as M_2-type are now further divided into M_2 and M_3 subtypes (Bonner, 1989). An M_3 receptor has been suggested to be responsible for the presynaptic inhibition of the glutamate synaptic potential in rat brain (Sugita et al., 1991).

4. Noradrenaline

In the CNS, adrenoceptors are classified into three categories: α_1, α_2 and β receptors. Each category has further subtypes (see Summers and McMartin, 1993 for review). As far as the adrenoceptors concerned here, α_1 adrenoceptors are linked to the production of IP_3 via G_q proteins and phospholipase C, resulting in an increase in intracellular free calcium

(Berridge and Irvine, 1989), and are directly coupled to calcium influx (Han et al., 1987); α_1 adrenoceptors mediate a depolarization of which the ionic mechanism is unclear yet. There is some evidence indicating that a decrease in the resting potassium conductance is in part involved in the α_1 response (VanderMaelen and Aghajanian, 1980; Legendre et al., 1988; Larkman and Kelly, 1992). The activation of α_1 adrenoceptors has also been shown to induce prolongation of afterhyperpolarizations (Freedman and Aghajanian, 1987). α_2 adrenoceptors are negatively coupled to adenylate cyclase through G_i proteins (Bylund, 1988). α_2 adrenoceptors act to cause an inhibition of neuronal firing by increasing potassium conductance (North and Yoshimura, 1984; Williams et al., 1985) and decreasing calcium conductance (Forscher et al., 1986).

Existing problems and the goals of present studies

At present, opioids are the most potent and selective agents available for the treatment of pain and thus are of great value clinically. The mechanism of opioid-induced analgesia has long been a important and interesting issue both physiologically and clinically. For the last two decades, our knowledge of opioid analgesia has grown considerably. It is now known that opioids produce analgesia largely by activating an endogenous pain-modulating networks, of which the NRM is a crucial component as a major output of the system. The clarification of the mechanisms underlying opioid analgesia relies largely on our understanding of the neuronal circuitry, the cellular mechanisms of opioid actions and the roles of these neurotransmitters in the system.

1. Problems

Previous anatomical studies have provided extensive information on the complexity of NRM composition. Behavioral and pharmacological studies have indicated the functional involvement of the NRM in nociceptive modulation. Electrophysiological studies have revealed functionally different groups of neurons involved in nociceptive transmission. Our current knowledge suffers from a lack of the connection between these findings, that is, from ignorance of the physiological identity of neurons containing a given transmitter and of actions of opioids and other transmitters (proven behaviorally to be effective) on these neurons. Moreover, in previous behavioral and electrophysiological studies, non-selective receptor agonists and antagonists were used for investigation. The presence of multiple receptor subtypes has made it much difficult to explain the functional significance of observations made with non-selective agonists, since the actions mediated by different receptor subtypes can not be distinguished. Additionally, the location of receptor subtypes on different neurons is a missing key to understand the role of the neurotransmitter studied.

Opioids consistently produce analgesia largely by activating descending inhibition from supraspinal structures including the NRM. The physiological mechanisms underlying opioid activation of the systems are unknown. Due to the general inhibitory actions of opioids and opioid-induced inhibition of inhibitory neurons in the CNS, it is hypothesized that opioids can activate the systems by disinhibition. There is no direct physiological evidence available to prove the hypothesis. In the NRM, the cellular mechanisms underlying the inhibition of activity in some neurons and the excitation in other neurons by analgesic opioids (applied systemically or locally) observed in previous in vivo studies are not known.

5-hydroxytryptamine is a major neurotransmitter in the NRM, particularly in the raphe-spinal descending systems, and may play an important role in antinociceptive function of the NRM. Previous studies failed to provide physiological evidence indicating the

functional role of the 5-HT-containing neurons in the NRM in opioid analgesia. This is due, at least in part, to the inability to characterize the actions of opioids and 5-HT on identified serotonergic neurons in physiological studies. The inconsistent effects of 5-HT on both neuronal activity and nociceptive transmission in the NRM from previous physiological and behavioral studies may result largely from the presence of functionally distinct groups of neurons and multiple 5-HT receptor subtypes which mediate opposite (excitation and inhibition) cellular responses. The physiological mechanisms of 5-HT-induced inhibition and excitation in activity of the NRM cells are to be clarified.

Acetylcholine has modulatory actions on the activity of pain-modulating neurons in the NRM, as suggested by previous studies. Relatively little is known about the role of this putative transmitter in the NRM such as the receptor subtypes, the receptor locations (presynaptic and postsynaptic) and the mechanisms involved in its cellular actions. The functional significance of cholinergic actions on different groups of the NRM cells in the NRM-mediated antinociception needs to be determined.

Noradrenaline may function as a neurotransmitter released from presynaptic noradrenergic terminals that tonically control the excitability of the neurons in the NRM, as indicated by previous electrophysiological and behavioral studies. It has been demonstrated that both α_1- and α_2-adrenoceptors are involved. The ionic mechanism of α_1-adrenoceptor-mediated depolarization has not yet been completely understood. Like other transmitters in the NRM, the noradrenergic actions on the excitability of different groups of neurons and their significance in nociceptive modulation by the NRM remain to be elucidated.

2. Goals of present studies

The brain slice has many advantages in studies on the cellular actions of opioids and neurotransmitters. It allows the characterization of the cellular mechanisms underlying the

actions with intracellular recordings from the neurons. The local neuronal circuitry in the slice preparation remains intact, allowing studies on presynaptic actions. Intracellular recordings in a brain slice also enable recovering of the neuron studied and identifying of the neurotransmitter it contains with subsequent immunohistochemistry.

In this series of studies in vitro, a brain slice preparation containing the NRM was developed and intracellular recordings with microelectrodes were made from NRM neurons in the preparation. The slice was cut from rat brains and maintained in a chamber through which flowed physiological saline at 37 C°. Effects of a variety of compounds on the neuronal excitability were examined by adding the compound into the perfusing solution. Focal electrical stimulation through the stimulating electrodes placed in the slice was used to evoke synaptic potentials. Membrane currents were studied by using single electrode voltage clamp techniques. In some experiments, whole cell recordings with a glass pipette were made from dorsal raphe neurons in slice preparations to study the ionic mechanisms of NA-mediated depolarization on raphe neurons.

The specific goals were as following:

1. Characterization of the electrical membrane properties of NRM neurons, including resting membrane potentials, resting membrane conductance, action potentials and spontaneous firing pattern.
2. Characterization of evoked synaptic potentials and pharmacological determination of the neurotransmitters that mediate the synaptic potentials by using selective receptor agonists and antagonists.
3. Examination and characterization of direct actions of opioids, 5-HT, ACh and NA on both the resting membrane potentials and each identified synaptic potential, thereby determining both presynaptic and postsynaptic actions of these compounds on neuronal excitability.
4. Clarification of the ionic mechanisms underlying any postsynaptic effects by using single electrode voltage clamp or whole cell recording techniques.

5. Identification of the receptor subtypes that mediate any presynaptic actions on synaptic potentials and postsynaptic actions on resting membrane potentials using pharmacological methods including Schild analysis with selective agonists and antagonists.
6. Determination of the relationships between the actions of these compounds and the intrinsic electrical properties of NRM neurons. The physiological significance of these actions and relationships in antinociceptive function of the NRM were considered.

By achieving above goals, it was hoped that our knowledge would be expanded concerning the mechanisms underlying opioid activation of the descending inhibition systems and the physiological roles of these neurotransmitters within the neural circuitry in the NRM and in opioid analgesia.

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Opioid actions on single nucleus raphe magnus neurons from rat and guinea pig *in vitro*

By: Z. Z. Pan, J. T. Williams and P. Osborne

From: Vollum Institute
Oregon Health Sciences University
3181 SW Sam Jackson Park Road
Portland, OR 97201, USA

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SUMMARY

1. Intracellular recordings were made from neurons of the nucleus raphe magnus (NRM) from rat (n=128) and guinea pig (n=115). Two types of cells were found in each, primary (103 in rat, 27 in guinea pig) and secondary cells (25 in rat, 88 in guinea pig).

2. Primary cells had input resistances of 186 ± 9 MΩ (n=9) in rat and 255 ± 50 MΩ (n=11) in guinea pig. The action potential in each was about 1.5 ms in duration. Synaptic potentials were evoked by focal electrical stimulation and consisted of a both γ–amino butyric acid (GABA) and excitatory amino acid components.

3. Morphine, [Met^5]enkephalin (ME) and [D–Ala^2,N–Me–Phe^4,Gly^5–ol]enkephalin (DAMGO) depressed the amplitude of the GABA–mediated synaptic potential by a maximum of 50–65% and had little effect on the excitatory amino acid–mediated synaptic potential. There was no effect of these opioids on the resting membrane potential or input resistance of primary cells in rat or guinea pig.

4. Secondary cells had short duration action potentials (< 1ms) and an input resistance of 354 ± 47 MΩ in rat (n=6) and 290 ± 40 MΩ in guinea pig (n=15). The synaptic potential observed in the cells of this group was mediated by activation of only excitatory amino acid receptors.

5. ME hyperpolarized and/or abolished the spontaneous firing in 16 out of 24 neurons in the secondary group from rat and 80 of 84 neurons from guinea pig. ME induced an outward current at –60 mV that reversed polarity at potentials more negative than –92 ± 3 mV in rat (n=6) and –98 ± 2 mV in guinea pig (n=18). The reversal potential of the opioid current was shifted to less negative potentials when the external potassium concentration

was increased, as predicted by the Nernst equation.

6. The morphology of the two types of cells were distinguishable in that primary cells were oval (29x18 μm in rat; 36x19 μm in guinea pig) with 2 to 4 thick tapering dendrites that branched within 50 μm from the cell body. Secondary cells were generally round or oval (about 24x13 μm in rat; 27x17 μm in guinea pig) with 2 to 5 thin (non–tapering) dendrites.

7. The results suggest that opioids increase the activity of a population of NRM neurons by presynaptic depression of GABA–mediated inhibitory input. This could be one of the mechanisms by which opioids modulate the descending inhibition from the NRM in the endogenous pain–modulating system.

INTRODUCTION

Nucleus raphe magnus (NRM), located in rostral medulla, has received much attention because of the involvement of its spinal projection in the endogenous pain modulating system of the brain (Mayer & Price, 1976; Basbaum & Fields, 1984). Previous studies in rat and cat have shown that antinociception can be produced when the neurons in the NRM are activated. For example, stimulation in the NRM inhibits spinal nociceptive neurons which were activated by noxious stimuli (Duggan & Griersmith, 1979; Giesler, Gerhart, Yezierski, Wilcox & Willis, 1981; Gebhart, 1986; Light, Casule & Menetrey, 1986). Stimulation in periaqueductal gray (PAG) caused inhibition of spinal nociceptive neurons and excited neurons in the NRM (Behbehani & Fields, 1979; Mason, Strassman & Maciewicz, 1988). The descending inhibitory projection of the NRM is also involved in opioid induced analgesia, for example, damage of NRM or its spinal projection significantly reduces the antinociception produced by systemic opioids or micro–application of opioids into PAG or NRM (Basbaum & Fields, 1984).

One major component of the descending projection from the NRM is inhibitory, suggesting that opioids produce analgesia by activating NRM neurons. Many NRM neurons, including those projecting to spinal cord, were excited by systemic administration or microinjection of opioids to PAG (Anderson, Basbaum & Fields, 1977; Fields & Anderson, 1978). It should be noted that most studies also report that a significant portion of NRM cells were inhibited by opioids (Deakin, Dickenson & Dostrovsky, 1977; Fields & Anderson, 1978; Toda, 1982, Duggan & North, 1983; Chiang & Pan, 1985). The role that these neurons play in descending pain modulation and opioid analgesia is not known.

Little is known about the mechanism by which opioids activate some NRM neurons and inhibit others (Duggan & North, 1983; Basbaum & Fields, 1984). In the present study, the membrane properties and responses to opioids of NRM neurons were studied in the slice preparation. An abstract of some of this work has been presented (Pan & Williams,

1989a).

METHODS

Intracellular recordings were made from NRM cells in slices of rat and guinea–pig medulla. The methods employed were similar to those published previously for the dorsal raphe (Pan & Williams, 1989b). Briefly, brain slices (300 μm) were cut in a vibratome in cold (4°C) physiological saline. Slices (coronal) were taken from the level of facial nerve where the facial nucleus was largest. A single slice was placed in a tissue bath through which flowed physiological saline (1.5 ml/min) at 37°C. The content of the physiological saline solution was (mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 11; $NaHCO_3$, 25; gassed with 95% O_2, 5% CO_2 at 37°C.

The area of the NRM was recognized in the slice as a triangular area in the midline just above the pyramidal tracts. Neurons were penetrated with glass microelectrodes filled with potassium chloride (2 M) having a resistance of 40–80 MΩ. Membrane currents were recorded with a single–electrode voltage–clamp amplifier (Axoclamp 2A) using switching frequencies between 3 and 6 kHz. The setting time of the clamp following a 10–mV step was typically 3–5 ms. Steady–state current–voltage (I–V) plots were constructed directly on an x/y plotter using a slow depolarizing ramp potential. The speed of the ramp (1 mV/s) was sufficiently slow to give the same current as that measured at thetermination of a 2–s step.

Bipolar tungsten stimulating electrodes were placed in the slice lateral (100–300 μm) and dorsal (100–300 μm) to the NRM. Single electrical stimuli (0.05–0.4 ms) of constant voltage were used to evoke synaptic potentials. Such local stimulation was expected to

activate all the fibers in this area. Placement of the stimulating electrode and adjustment of the stimulus voltage were made so that no antidromic or directly activated action potentials were evoked.

Drugs were applied by superfusion. The following drugs and salts were used: 6–cyano–2,3–dihydroxy–7–nitro–quinoxaline (CNQX, Tocris Neuramin), DL–2–amino–5–phosphonovaleric acid (APV), kynurenic acid, (–)–bicuculline methiodide, [Met^5] enkephalin (ME), [D–Ala^2,N–Me–Phe^4,Gly^5–ol] enkephalin (DAMGO), [D–Pen^2,D–Pen^5] enkephalin (DPDPE), γ–amino butyric acid (GABA) (All from Sigma) and naloxone (Endo). Numerical data are presented as means ± S.E. of the means. Comparisons of experimental data was carried out using a paired T–test.

In some experiments, the recording electrode was filled with a solution containing KCl (2 M), Tris (50 mM, pH=7.2) and biocytin (2%, Sigma). Usually diffusion of biocytin alone was sufficient to fill the cell although sometimes hyperpolarizing current (20–60 pA) was used to speed diffusion (Horikawa & Armstrong, 1988). Recordings for at least 20 min were required to obtain sufficient staining. Only one cell was labeled in each slice. The properties of the electrodes, the cells and the responses to drugs using this electrode solution was not distinguishable from KCl (2 M) filled electrodes. After the experiment, the slice was fixed (2% formaldehyde/15% picric acid in 0.1 M phosphate buffer, pH 7.0) overnight and then washed successively in ethylalcohol (80%), dimethyl sulfoxide (DMSO) and three times in phosphate buffer solution (PBS, 0.1 M, pH 7.2, 15–20 min each). The slice was then incubated with fluorescein isothiocyanate conjugated streptavidin (1:300, Jackson Immunoresearch Lab) overnight in a humid chamber and washed with PBS 3 times. The slices were mounted in biffered glycerol and examined using a fluorescence microscope.

RESULTS

TWO TYPES OF CELLS IN RAT

Intracellular recordings were made from a total of 128 cells in rat. Two types of cells were distinguished, 103 of the 128 cells were called primary cells and the other 25 cells were termed secondary cells.

Primary cells The cells in this group had resting membrane potentials between –50 and –75 mV and were generally not spontaneously active (8 of 104 fired action potentials spontaneously). The input resistance measured near the resting membrane potential was 186 ± 9 MΩ (range 143–235 MΩ, n=14). Action potential duration measured at the threshold for activation was between 1–1.8 ms (Figure 1A).

Focal electrical stimulation evoked a synaptic potential with a latency of 0.5–3 ms that peaked within 4–10 ms and had a total duration less than 200 ms. The amplitude of the depolarization was dependent on the stimulation intensity and action potentials could be evoked at the peak of larger depolarization. All cells were hyperpolarized by passing current through the recording electrode to hold the membrane potential at about –70 mV in order to obtain a 10–15 mV depolarization without activating action potentials. This synaptic potential was completely blocked by tetrodotoxin (TTX, 1 μM), $CoCl_2$ (2 mM) or solutions containing no calcium.

In all cells tested (n=61) in this primary group, the synaptic potential induced by the stimulation was depressed in amplitude by excitatory amino acid receptor antagonists (APV, 10 μM and CNQX, 10 μM). The amplitude of the synaptic potential that remained was 73 ± 7% (n=8) of the total depolarizing synaptic potential (Figure 2A). That synaptic potential had a slower rate of rise and was completely blocked by bicuculline (30 μM, Figure 2A). This $GABA_A$–mediated synaptic potential was depolarizing (from –70 mV) in these experiments because of the use of KCl filled electrodes. The reversal potential of

$GABA_A$ receptor mediated current induced by muscimol (1–3 μM) was –46 ± 3 mV (n=5).

The amplitude of the excitatory amino acid component, studied in the presence of bicuculline (30 μM), was generally smaller than the GABA component when the same stimulus strength was employed (46 ± 5% of the total synaptic potential, n=5).

Secondary cells The resting membrane potential in this group of cells was between –50 and –68 mV with input resistance of 354 ± 47 MΩ (range: 200–571 MΩ, n=6) and the action potential duration was <1 ms (Figure 1B). Spontaneous action potentials were recorded in 7 of the 25 cells. The synaptic potentials were completely blocked by APV (10 μM) and CNQX (10 μM) in all cells tested (n=10). Bicuculline (30 μM) had no effect on the synaptic potential in these cells. Unlike the primary cell group, these cells appeared to receive mostly excitatory amino acid input (Figure 2B).

TWO TYPES OF CELLS IN GUINEA PIG

In guinea–pig, 27 of 115 cells were the primary cell type and 88 cells were the secondary type. Unlike those in rat, about 70 percent of all cells (both primary and secondary) fired action potentials spontaneously.

Primary cells The primary cells in guinea–pigs had characteristics similar to those of primary cells in rats. That is, the action potential was >1 ms in duration and there were synaptic potentials mediated by both GABA and an excitatory amino acid. The 'resting' membrane potential varied from –52 to –70 mV and the input resistance was 255 ± 50 MΩ (range: 105–660 MΩ, n=11).

Secondary cells Secondary cells in the guinea–pig were similar to those found in rat in that they had action potentials of shorter duration (<1 ms) and a fast synaptic potential that was mediated by an excitatory amino acid. The input resistance of these cells was 290 ± 40

MΩ (range: 118–667 MΩ, n=15). The amplitude of synaptic potentials was 8.7 ± 0.4 mV in control, 10.9 ± 0.1 mV in bicuculline (30 μM) and 0.7 ± 0.2 mV in bicuculline plus APV (30 μM) and CNQX (10 μM), (n=4).

ACTIONS OF OPIOIDS IN RAT

Primary cells The synaptic potential was depressed by opioids. The actions of opioids on the excitatory amino acid and $GABA_A$ synaptic potentials were studied separately. No change in the resting membrane potential or input resistance was caused by ME, DAMGO or DPDPE in these cells including those that were firing spontaneously.

$GABA_A$ synaptic potential. These experiments were carried out in the presence of CNQX (10 μM) and APV (10 μM) or kynurenic acid (500 μM). ME, DAMGO and morphine depressed the amplitude of the GABA–mediated synaptic potential in every cell tested (Figure 3A). The inhibition induced by ME was dependent on the concentration applied having a threshold of 100nM and an EC_{50} of about 1 μM. The maximum inhibition was about 50% of the control at 30 μM (Figure 3B).

The selective μ-opioid receptor agonist, DAMGO, caused a concentration dependent inhibition of the GABA–mediated synaptic potential (Figure 4A). The threshold concentration was 10 nM, with an EC_{50} of about 70 nM and the maximum inhibition (at 1 μM) was 58 ± 6% (n=5). The concentration response curve for DAMGO was shifted to the right in the presence of naloxone (100 nM, Figure 4B). The EC_{50} for DAMGO in naloxone was 4.8 μM which was about 65 fold larger than that in control. The estimated naloxone K_d from this single shift (Kosterlitz & Watt, 1968) was 1.5 nM. DPDPE (300 nM – 1 μM), a selective δ-opioid receptor agonist, had no effect on the GABA–mediated synaptic potential (control = 12.3 ± 0.6 mV, DPDPE (300 nM) = 12.1 ± 0.8 mV; n=6; $p>0.05$).

Excitatory amino acid synaptic potential. The excitatory amino acid–mediated synaptic potential was studied in the presence of bicuculline (30 μM). The average amplitude was 7.6 ± 1.1 mV (n=7) in control and 6.7 ± 1.1 mV (n=7) in the presence of ME (10 μM, Figure 5A). The small inhibition of the synaptic potential, a reduction to 90 ± 2% of the control (n=7), was significant ($p<0.02$) but did not recover after washout.

GABA applied exogenously. The depolarization produced by GABA applied bysuperfusion was not affected by either ME (10 μM) or DAMGO (300 nM) in each of 5 cells tested (Figure 5B). Superfusion with bicuculline (30 μM) blocked most of the GABA induced depolarization.

Secondary cells ME (10 μM) or DAMGO (300 nM) caused a hyperpolarization or inhibition of 16 out of 24 secondary cells tested. The membrane potential of the remaining cells was not affected by opioids. ME (10 μM) caused a 6 ± 1 mV (n=12) hyperpolarization of secondary cells (Figure 6A). The spontaneous firing of the other 4 neurons was completely blocked. Under voltage clamp ME (10 μM) caused an outward current that reversed polarity at –92 mV ± 3 mV (n=6). In a single experiment the reversal potential of the ME induced current was shifted to –85 and –70 mV as the external potassium was increased to 6.5 and 10.5 mM, respectively. None of the opioids tested produced a change in the excitatory amino acid–mediated synaptic potential in these cells.

EFFECTS OF OPIOIDS IN GUINEA PIG

Primary cells As was found in rat, opioids had no effect on the membrane potential and the GABA–mediated synaptic potential was depressed. The amplitude of the GABA synaptic potential was 12.0 ± 1.4 mV in control and 5.1 ± 0.6 mV in the presence of DAMGO (300 nM, n=8), whereas DPDPE was without effect (7.8 ± 0.3 in control, 7.6 ± 0.2 in DPDPE (300 nM)). As in the rat, DAMGO (300 nM) also caused a small reduction in the amplitude

of the excitatory amino acid mediated synaptic potential (from 8.7 ± 1.1 mV to 7.5 ± 1.1 mV, n=4).

Secondary cells In 80 of 84 secondary cells ME (10 μM) caused a hyperpolarization (Figure 6B). The amplitude of the hyperpolarization induced by ME (10 μM) was 9 ± 1 mV (n=64), DAMGO (300 nM) was 10 ± 1 mV (n=15) and DPDPE (300 nM) produced a 3 ± 1 mV hyperpolarization (n=12).

Under voltage clamp (at –60 mV), an outward current was induced by ME or DAMGO with an increase in conductance. The current reversed polarity at –103 ± 0.7 mV (n=7) in a solution with normal potassium concentration. As the potassium concentration in the perfusing solution was increased to 6.5 mM and 10.5 mM, the reversal potential shifted to –80 ± 3 mV and –67 ± 4 mV, respectively (n=7, Figure 6C). These shifts in reversal potential with extracellular potassium concentration were in accordance with the relationship predicted by Nernst equation (slope = –58.5 ± 1.2, n=7).

INTRACELLULAR STAINING

The cellular morphology of raphe magnus neurons has been described in rat (Steinbusch & Nieuwenhuys, 1983), rabbit (Felten & Cummings, 1979), and cat (Edwards, Johnston, Poletti & Foote, 1987). In rat, two basic cell types could be distinguished: medium–sized cells (approx. 21 μm long) that were multipolar and oval in shape; and large cells (approx. 35 μm long) that were fusiform. In the present study, 20 electrophysiologically characterized cells in rat (12 primary and 8 secondary) and 15 cells in guinea pig (9 primary and 6 secondary) were filled with biocytin and subsequently visualized.

Primary cells In rat these cells had an average length of 29 μm (range: 22–47 μm) and width of 18 μm (range: 13–28 μm). Most cells were oval (9 of 12) 2 others were bipolar and 1 was tripolar. Most cells had 2–4 primary dendrites that were large in diameter near the cell body and tapered rapidly within 20–40 μm of the cell body. These dendrites

branched within 20–50 μm of the cell body giving rise to multiple secondary dendrites (Figure 7A). The dendritic tree of most cells tended to be orientated in the medio–lateral direction, this was most strongly evident for the bipolar cells.

In guinea pig 9 primary cells were stained. These cells were larger than those in rat having an average length of 36 μm (range: 22–53 μm) and width of 19 μm (range: 16–27 μm) and were very similar in morphology to the rat primary cells (Figure 7C).

Secondary cells Secondary cells in the rat tended to be smaller than primary cells with an average length of 24 μm (range: 17–31 μm) and width of 13 μm (range: 11–19 μm). All 8 cells were round or oval in shape and had 3–5 primary dendrites (Figure 7B). In these cells the primary dendrites did not taper in diameter within 20–40 μm of the cell body as was observed in the primary cells. Few secondary dendrites were seen to arise from the primary dendrites within 50–100 μm of the cell body. The processes did not appear to have any particular orientation.

In guinea pig all 6 cells were round or oval with an average length of 27 μm (range: 20–38 μm) and width of 17 μm (range: 13–19 μm) (Figure 7D). These cells like those observed in rat had 2 to 4 primary dendrites with little or no taper in the diameter as they left the cell body. Unlike the rat, numerous secondary dendrites were seen within 30–60 μm of the cell body.

DISCUSSION

TWO CELL TYPES

Two distinct types of NRM cells were found in both rat and guinea pig. In rat, the primary cell group made up the major population of neurons. The major differences between the two populations were in the type of synaptic potentials and the response to opioids. In primary cells there was a large GABA–mediated synaptic potential, whereas little or no GABA–mediated synaptic potential was found in secondary cells. In both cell types, excitatory amino acid–mediated synaptic potentials were observed. The membrane potential of primary cells was not significantly changed by opioids, while the spontaneous activity of most secondary cells was inhibited and/or the membrane potential was hyperpolarized by opioids.

Differences in morphology were also noted. Primary cells tended to be larger and showed extensive dendritic branching in the region of the cell body. Secondary cells seemed smaller than primary cells and had few secondary dendrites near the cell body (in rat). The major difference between these cell types was the shape of the primary dendrites as they left the cell soma. The dendrites of primary cells arose from thick extensions of the cell soma whereas no such thickenings of the soma were observed in secondary cells.

One species difference was the relative abundance of secondary cells in the guinea pig, although, this difference may be a sampling error resulting from the use of microelectrodes. Another species difference was the morphology; both types of cells were larger in guinea pig and a greater number of dendritic branches were found near the cell soma of secondary cells in guinea pig. The difference in size may account for the increase in the number of recordings from guinea pig secondary cells. In any case, the two cell types in each species seem similar in all other respects and suggests that these two cell types could be functionally common cell populations. Correlations have been made between NRM cell types from rat and cat in control of descending pain modulation (Fields, Barbaro &

Heinricher, 1988), but no similar studies hve been made in guinea pig.

ACTIONS OF OPIOIDS, PRIMARY CELLS.

In the rat and guinea pig, opioids inhibit the GABA–mediated synaptic potential by about 50% in the primary cells of the NRM. DAMGO, a selective μ–opioid receptor agonist, was effective, whereas DPDPE, a selective δ–opioid receptor agonist, was not. In addition, in rat, the K_d for naloxone was estimated to be about 1.5 nM suggesting an action on a μ-opioid receptor. These findings suggest that the opioid induced inhibition of the GABA–mediated synaptic potential was mediated by a μ–opioid receptor. Previous autoradiographic studies have described μ–opioid binding in the NRM of rat (Bowker & Dilts, 1988; Mansour, Khachaturian, Lewis, Akil & Watson, 1988). Since neither the resting membrane potential of the cell nor the response of the cell to exogenously applied GABA was changed by ME or DAMGO, the inhibition of the GABA–mediated synaptic potential by opioids probably results from presynaptic inhibition of GABA release.

The GABA–mediated synaptic potential was the predominant 'fast' synaptic potential in primary neurons of the NRM under the conditions of these experiments. It has been shown in the dorsal raphe nucleus that the GABA–mediated synaptic potential was inhibitory at resting membrane potentials when non–chloride electrodes were used and reversed its polarity at about –70 mV (Pan & Williams, 1989b). Moreover, exogenously applied GABA caused aninhibition of NRM neurons *in vivo* (Nishikawa & Scatton, 1985). Therefore, opioids would be expected to increase the activity of primary neurons by disinhibition. Some of these primary neurons may project to the dorsal horn of the spinal cord. Consequently, descending inhibition from the NRM could be facilitated by opioids. This may be one of the mechanisms involved in the opioid produced antinociception.

Two physiologically identified group of cells have been described in the NRM *in vivo* based on their response to a noxious stimulus, OFF– and ON–cells (Fields, Vanegas,

Hentall & Zorman, 1983; Fields, Barbaro & Heinricher, 1988). In response to a noxious stimulus, the firing of OFF–cells ceased just prior to the tail–flick. Opioids prevented this inhibition in firing. ON–cells were defined by a burst of firing just before the tail flick in response to a noxious stimulus. Opioids blocked this burst of activity. The effect of opioids on the activity of primary cells described here correlates with OFF–cells. In fact, excitation of OFF–cell by opioids was hypothesized to result from disinhibition because of the lack of convincing evidence for a direct excitatory opioid effect. The cells that were excited by opioids were thought to cause descending inhibition of spinal nociceptive dorsal horn neurons (Fields, Barbaro & Heinricher, 1988).

ACTIONS OF OPIOIDS, SECONDARY CELLS.

ME or DAMGO hyperpolarized most secondary neurons. DPDPE (300 nM) had no effect in the rat and only weakly hyperpolarized neurons in the guinea pig. The results suggest that activation of μ–opioid receptors caused a hyperpolarization in both species, although in guinea pig, a δ–opioid receptor mediated hyperpolarization has not been ruled out. In each species, the hyperpolarization resulted from an increase in potassium conductance.

This group of cells is similar in the response to opioids to the functionally defined ON–cells (Fields, Barbaro & Heinricher; 1988). These cells may function as local circuit interneurons (presumed to be GABA containing) or as a group of descending excitatory neurons which facilitate the nociceptive transmission in the spinal cord and are inhibited by opioids to cause analgesia (Fields, Barbaro & Heinricher; 1988).

Inhibition of local inhibitory neurons in the central nervous system has been suggested to be a common mechanism of opioid action (Nicoll, Alger & Jahr; 1980). Recently, interneurons that release GABA onto hippocampal pyramidal cells were found to be hyperpolarized by opioids (Madison & Nicoll, 1988). An inhibitory GABA input onto the NRM neurons involved in the regulation of nociceptive threshold has been suggested (Drower & Hammond, 1988). In addition, numerous GABA–transaminase staining

neurons have been identified immunohistochemically in the rat NRM (Nagai, Maede, Imai, McGeer & McGeer, 1985). No distinct morphology of those neurons was noted. A population of those (GABA–containing) neurons may be sensitive to opioids and thus mediate the presynaptic inhibition of GABA release onto primary cells.

In summary, we have found that the GABA–mediated synaptic potential in the primary cells of the NRM in rats and in guinea–pigs was reduced in amplitude by opioids acting on μ–opioid receptors. Reduction of this synaptic potential would be expected to increase activity of these neurons by disinhibition. In addition, many secondary neurons were directly inhibited by opioids through an increase in potassium conductance.

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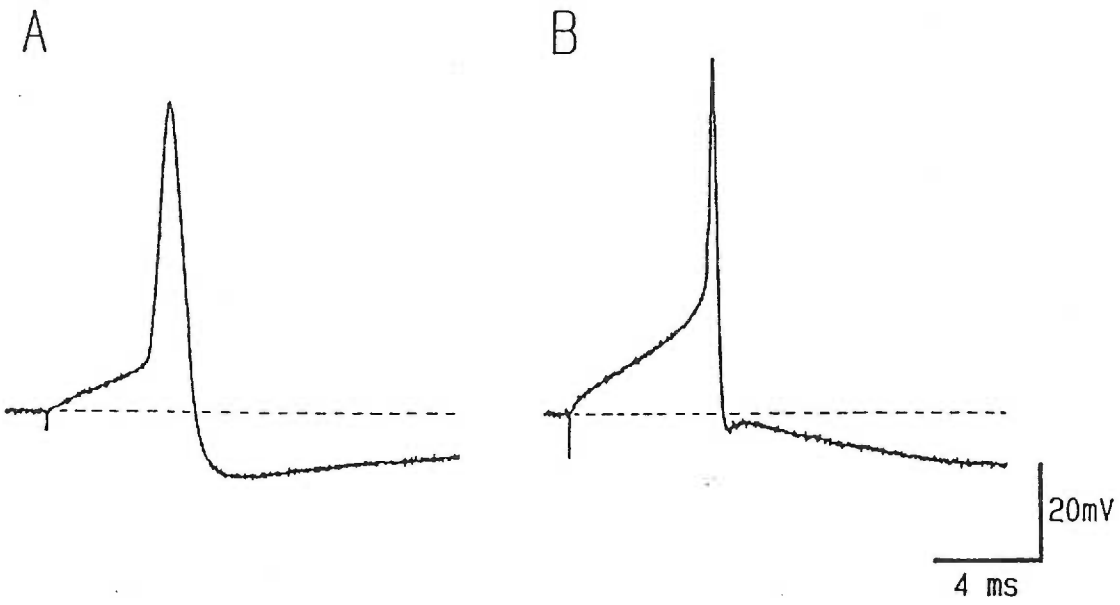

Figure 1. Action potentials from two cell types in rats, A, a primary cell and B, a secondary cell. Depolarizing electrotonic potentials were applied to evoke the action potential from –65 mV.

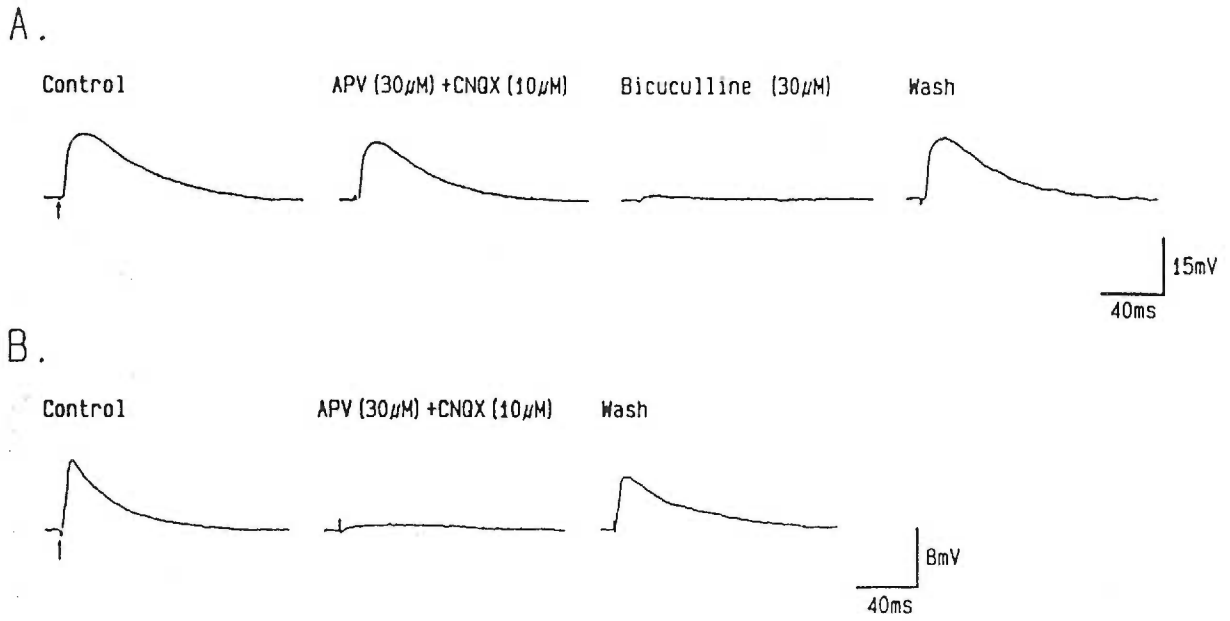

Figure 2. Separation of the GABA and excitatory amino acid synaptic potentials in two types of cells from rats. KCl filled electrodes were used in each. A, a primary cell where a small reduction in the synaptic potential amplitude was produced by superfusion with APV and CNQX. Addition of bicuculline to the solution containing APV and CNQX completely blocked the remaining synaptic potential. B, a secondary cell. The synaptic potential was almost completely abolished by APV and CNQX. Each trace is the average of 4. Small arrows indicate the stimulus artifact.

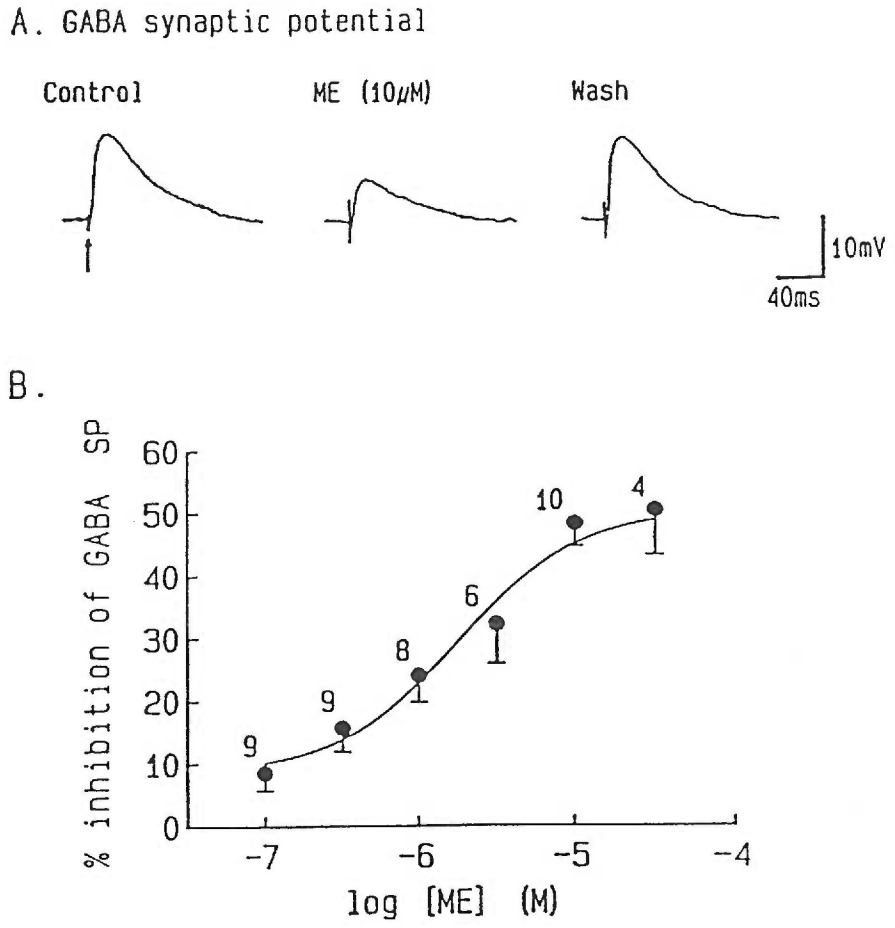

Figure 3. Opioids inhibit the GABA–mediated synaptic potential in rat primary cells. A, GABA–mediated synaptic potential (average of 4) in control (left), in the presence of ME (10 μM) (middle) and after wash out ME (right). No change in the resting membrane potential was produced by ME. B, concentration dependence of the ME induced inhibition of GABA–mediated synaptic potentials. Percent inhibition of GABA synaptic potential (SP) plotted as a function of the ME concentration. Numbers beside each point indicate the number of cells tested at that concentration. The amplitude of the GABA synaptic potential in control was 13.4 ± 1.0 mV (n=10). All these experiments were carried out in the presence of APV and CNQX to block excitatory amino acid receptors.

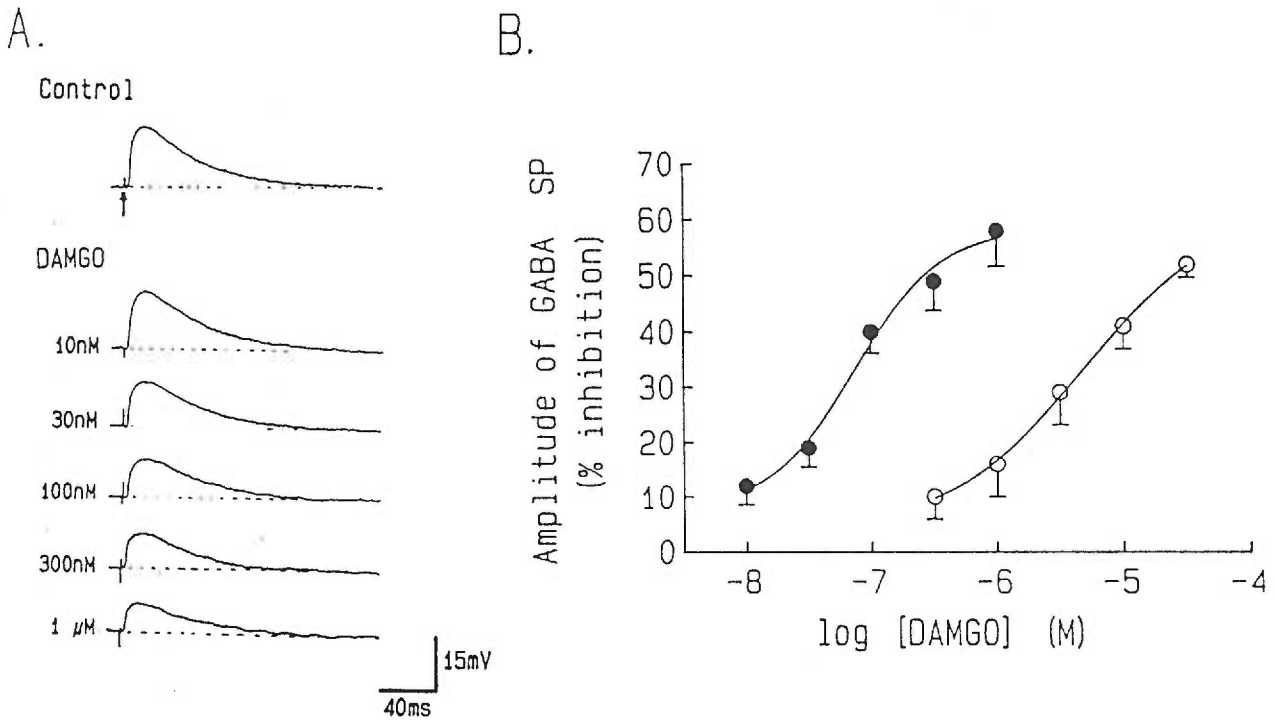

Figure 4. Concentration–dependent inhibition of GABA–mediated synaptic potentials by DAMGO in primary cells from rats. Experiments were carried out after blockade of excitatory amino acid receptors with APV and CNQX. A. DAMGO (10 nM – 1 μM) caused an inhibition of the GABA–mediated synaptic potential. Each trace is the average of 4. B. concentration–dependence curve of the % inhibition of the GABA synaptic potential as a function of the DAMGO concentration, in control (filled circles) and in the presence of naloxone (100nM) (open circles). The EC_{50} for DAMGO was 74 nM in control and 4.8 μM in naloxone.

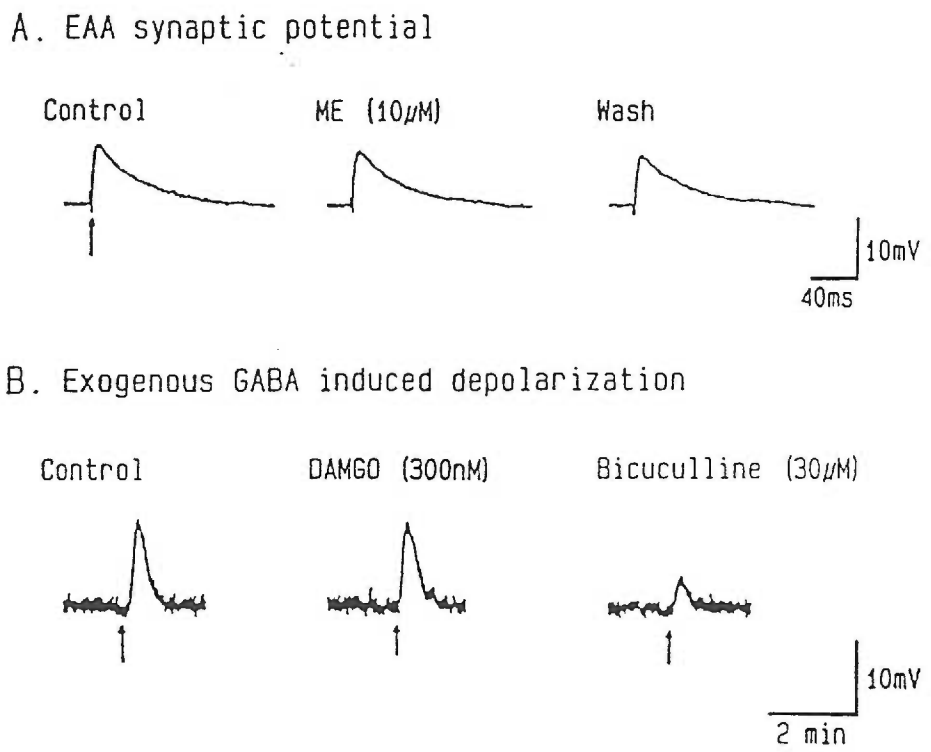

Figure 5. Opioids had little effect on the excitatory amino acid–mediated synaptic potential and the depolarization induced by exogenously applied GABA in rat primary cells. A. Recordings from a primary cell in the presence of bicuculline (30 μM) to block the $GABA_A$ receptors. Excitatory amino acid (EAA) mediated synaptic potentials (average of 4) before (left), during (middle) and after (right) application of ME (10 μM). The resting membrane potential was not changed by ME. B. Depolarization induced by exogenously applied GABA was not changed by DAMGO. Left: Superfusion (indicated by the arrow) with GABA (10 mM) for 4 seconds produced a depolarization. Middle: DAMGO (300 nM) had no effect on the GABA mediated depolarization. Right: The GABA mediated depolarization was reduced by bicuculline (30 μM).

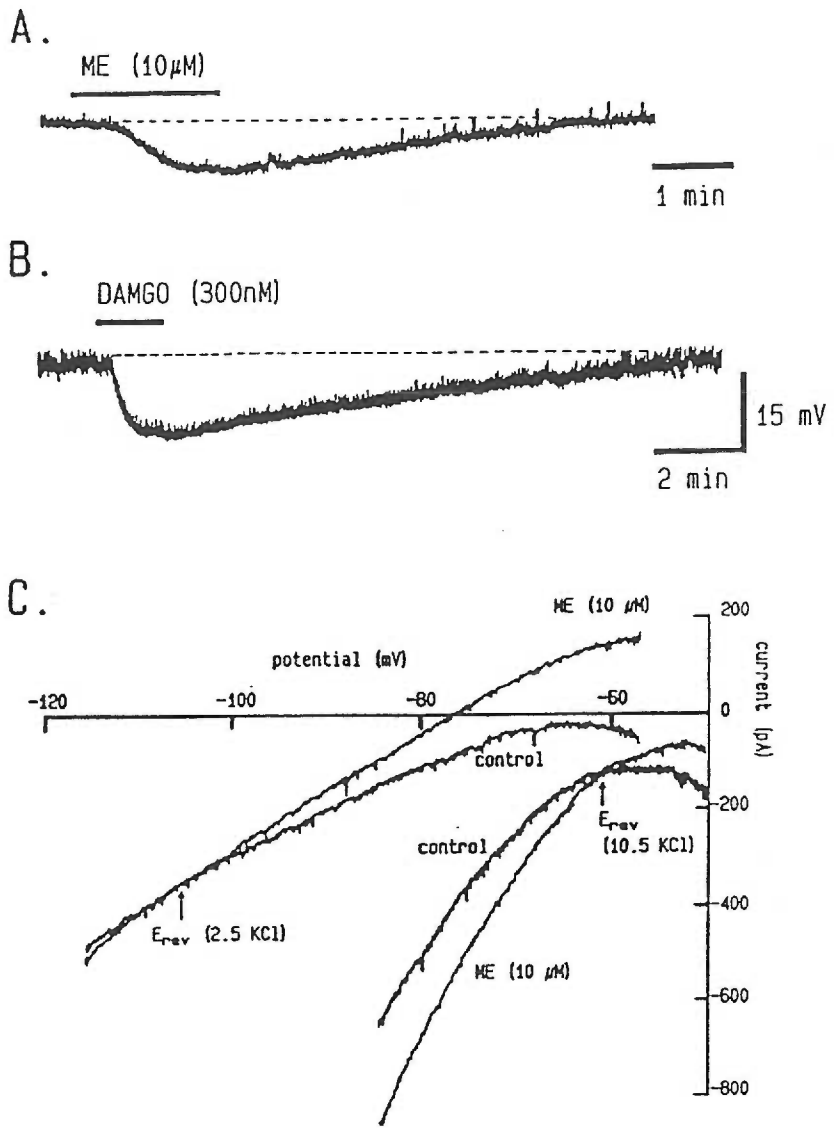

Figure 6. Opioids hyperpolarized secondary cells by increasing potassium conductance. A, recording of membrane potential from a secondary cell in the rat. ME (10 μM) produced a hyperpolarization of 9 mV from –62 mV. B, in the guinea–pig DAMGO (300 nM) caused a 12 mV hyperpolarization from –70 mV (This cell was held at –70 mV by passing negative current through recording electrode). C. Steady–state current–voltage plots in the presence and absence of ME (10 μM) in two different concentrations of potassium from another secondary cell in the guinea–pig. The reversal potential was shifted from –105 mV in 2.5 mM potassium to –62 mV in 10.5 mM potassium.

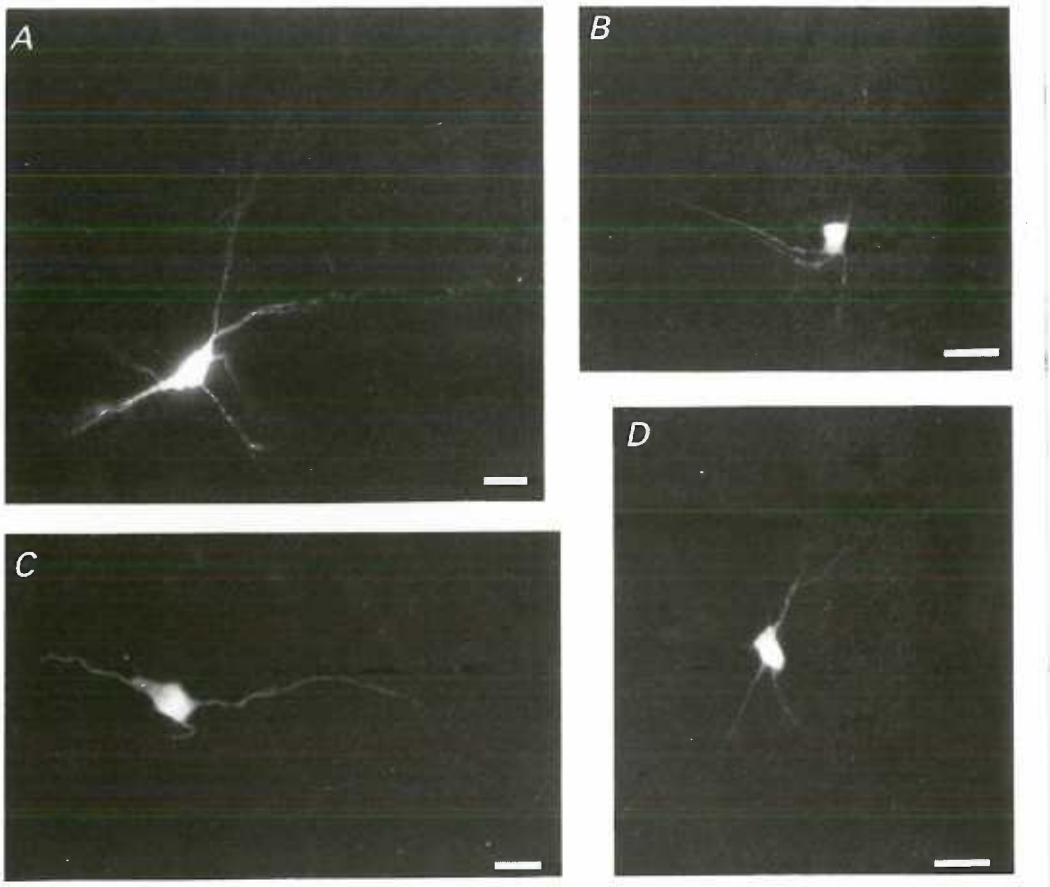

Figure 7. Representative NRM neurons filled with biocytin and labeled with FITC–conjugated streptavitin. A, a rat primary cell. B, a rat secondary cell. C, a guinea-pig primary cell. D, a guinea-pig secondary cell. Scale bars = 30 μm.

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MODULATION BY SEROTONIN OF THE NEURONS IN RAT NUCLEUS RAPHE MAGNUS IN VITRO

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Z.Z. Pan*, M.W. Wessendorf# and J.T. Williams*¤

* Vollum Institute, Oregon Health Science University, Portland, OR, 97201

\# Dept. of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, MN 55455, USA

¤ Corresponding author. FAX (503) 494-6972

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ABSTRACT

Nucleus raphe magnus contains a large population of raphe-spinal serotonergic neurons that are thought to be involved in descending control of pain transmission and the modulation of opioid analgesia. Intracellular recordings were made from nucleus raphe magnus neurons in the slice preparation. Cells were divided into two groups, primary and secondary cells, based on the action potential waveform and response to opioids, as previously reported. In some experiments, cells were filled with biocytin and 5-hydroxytryptamine (5-HT)-containing cells were identified immunohistochemically. Of the primary cells that were filled with biocytin, 93% stained for 5-HT; 90% of biocytin filled secondary cells were unlabeled for 5-HT. Previous studies have shown that primary cells are disinhibited by opioids; the finding that most primary cells are serotonergic suggests that at least some 5-HT contining neurons in the NRM are excited by opioid analgesics. 5-HT hyperpolarized cells in both primary and secondary cell groups. The 5-HT agonists, ((±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene HBr and 5-carboxamidotryptamine mimicked this action of 5-HT, indicating that the 5-HT_{1A}-subtype mediated this hyperpolarization. The hyperpolarization was mediated by an increase in potassium conductance that rectified inwardly. Local electrical stimulation of afferents evoked an inhibitory postsynaptic potential in primary cells. The inhibitory postsynaptic potential reversed polarity at the potassium equilibrium potential and was blocked by 5-HT_{1A}-receptor antagonists. It is proposed that the 5-HT_{1A} receptor on serotonergic primary cells may function as an autoreceptor to regulate the activity. The role of the 5-HT_{1A} receptor on non-serotonergic secondary cells is unclear.

INTRODUCTION

It is generally believed that opioids produce antinociception, at least in part, by activating bulbo-spinal inhibitory pathways originating from nucleus raphe magnus (NRM) and adjacent medullary structures[4]. Since NRM contains large population of raphe-spinal serotonergic cells[29] and dense serotonergic varicosities[31], it is of interest both to identify, physiologically, the 5-HT containing cells and to determine the role of serotonin (5-HT) as a neurotransmitter at the level of NRM.

Of particular interest is the effect of 5-HT itself on serotonergic neurons. Although, there is anatomical, pharmacological and behavioral evidence supporting the involvement of 5-HT in opioid-induced antinociception[15,19], contradictory findings have been reported regarding the effect of 5-HT on nociceptive threshold. Systemic administration or local microinjection of 5-HT or 5-HT receptor agonists into NRM produced either analgesia[17,20], hyperalgesia[5,22] or no effect[1]. The role of 5-HT in antinociception has been complicated by the finding that the analgesic action of opioids was reduced by systemic application of 5-HT receptor agonists[5,23]. The discrepancy could be attributed to many factors such as, the dose applied, the species studied, the type of nociceptive tests used and the existence of multiple 5-HT receptor subtypes which mediate opposing cellular responses[8,28]. There are also different groups of pain modulating cells within rostral medulla and within the NRM itself[15] that may account for the inconsistency.

In a previous study[26], two functionally different types of NRM cells were characterized: primary cells that are most often encountered and secondary cells that comprised about 20% of total cells studied in the NRM slice. Opioids decreased GABA-mediated synaptic transmission onto primary cells and thus could activate primary cells indirectly by

disinhibition. This has been proposed to be a possible mechanism for opioid activation of the descending inhibitory pathway from NRM. Activation of μ-opioid receptors directly hyperpolarized secondary neurons by increasing potassium conductance[26]. In view of their responses to opioids, the primary and the secondary cell groups were correlated respectively to the "Off" cells (cells which show an abrupt cessation in firing just prior to the tail-flick) and the "On" cells (cells which display a burst of activity just before the tail-flick) characterized in rostral medulla including NRM by Fields and colleagues[13,14]. Thus both primary cells and "Off" cells can be activated by opioids and may be involved in opioid-induced antinociception. Secondary cells and "On" cells are inhibited by opioids and could play a different role in nociceptive processing.

To understand the function of 5-HT in modulation of opioid analgesia through NRM, it is necessary to characterize the modulatory actions of 5-HT on electrophysiologically identifiable groups of cells and determine which group is serotonergic. The purpose of this study was to investigate the direct effects of 5-HT on primary and secondary cells in the NRM, that is, to characterize the ionic mechanism(s), identify the 5-HT receptor subtype(s) and determine which cell type(s) contained 5-HT. The possible contribution of 5-HT as a neurotransmitter in the neuronal circuitry within NRM and its role in the mediation of opioid analgesia is discussed. A preliminary account of this work has been presented as an abstract[27].

Experimental Procedures

Intracellular recordings were made from NRM cells in brain slices from adult Wistar rats. The methods employed were similar to those published previously[26]. Briefly, brain slices (300 μm thick) were cut in a vibratome in cold (4 °C) physiological saline. Two or three coronal slices were taken from the level of facial nerve in each rat brain. A single

slice was submerged in a tissue bath through which flowed physiological saline (1.5 ml/min) at 37 °C. The content of physiological saline solution was (mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; CaCl, 2.4; glucose, 11; $NaHCO_3$, 25; gassed with 95% O_2, 5% CO_2 at 37 °C.

The area of the NRM was recognized in the slice as a triangular area in the midline just above the pyramidal tracts. Neurons were penetrated with glass microelectrodes having a resistance of 40-70 MΩ. Membrane currents were recorded with a single-electrode voltage-clamp amplifier (Axoclamp 2A) using switching frequencies between 3 and 6 KHz. The setting time of the clamp following a 10 mV step was typically 3-5 ms. Steady-state current-voltage (I-V) plots were constructed directly on an x/y plotter using a slow depolarizing ramp potential. The speed of the ramp (1 mV/s) was sufficiently slow to give the same current as that measured at the termination of a 2 second voltage step.

Bipolar tungsten stimulating electrodes were placed in the slice lateral (100-300 μm) and dorsal (100-300 μm) to the NRM. Single constant current electrical stimuli (0.1-0.4 ms, 0.5-5 mA) were used to evoke synaptic potentials. Placement of the stimulating electrode and adjustment of the stimulus voltage were made so that no antidromic or directly activated action potentials were evoked. Drugs were applied by superfusion.

In most experiments, the recording electrode was filled with a solution containing KCl (2 M), Tris (50 mM, pH=7.2), and biocytin (2%, Sigma). Usually diffusion of biocytin alone was sufficient to fill the cell and sometimes hyperpolarizing current (20-60 pA) was used to speed diffusion. Recordings were maintained for at least 20 min to obtain sufficient staining. Only one cell was labeled in each slice. The properties of the electrodes and the responses of the cells to drugs using this electrode solution were not distinguishable from those filled with KCl (2 M). After the experiment, the slice was incubated in physiological saline containing tryptophan (100 μM) and pargyline (10 μM) for 30-60 min. The slice was then fixed in 2% formaldehyde/15% saturated picric acid in

0.1 M phosphate buffer at pH 7.0 overnight, washed thoroughly in phosphate buffer solution (PBS, 0.1 M, pH=7.2) at least three times for 20-30 min each and stored in PBS plus sucrose (5%).

To determine the serotonin content of biocytin filled cells, sections were stained immunohistochemically. Cryostat sections (30 μm) of slices were cut and incubated overnight in rabbit anti-5-HT that was diluted 1:1000 in PBS containing 0.3% Triton X-100 (PBS-Triton). Sections were washed in PBS and were then incubated for 1-2 hours at room temperature in PBS-Triton containing the following two reagents: donkey anti-rabbit IgG conjugated with the red fluorophore cyanine 3.18 (Jackson Immuno Research, West Grove, PA) diluted 1:600, and avidin conjugated with the blue fluorophore DAMC (Organon Technika-Cappel, Durham, NC) diluted 1:60. Sections were then washed in PBS and were coverslipped using a PBS-glycerin mounting medium. Staining in this manner resulted in biocytin-labeled neurons appearing blue and serotonin-labeled neurons appearing red.

Tissue was examined using an Olympus BH-2 microscope equipped with a 200 Watt mercury illuminator, a x40/0.85 n.a. dry D-planapo objective (Olympus), and a filter set modified especially for multicolor fluorescence microscopy (Leeds Precision Instruments, Golden Valley, MN). DAMC was visualized using a UG1 excitation filter and a 420-470 nm emission filter (Omega Optical, Brattleboro, VT). Cyanine 3.18 was visualized using a 541-551 nm excitation filter and a 573-608 nm emission filter (Omega Optical, Brattleboro, VT). Photomicrographs were taken using Kodak 2415 Technical Pan film hypersensitized using forming gas (H_2/N_2 - 8/92%)[30].

MATERIALS. The following drugs and salts were used: DL-2-amino-5-phosphonovaleric acid (AP-5, Sigma), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX, Tocris Neuramin), kynurenic acid (Sigma), (-)bicuculline methiodide (Sigma), prazosin (Pfizer),

[Met^5] enkephalin (ME, Sigma), [D-Ala^2,N-Me-Phe^4,Gly^5-ol] enkephalin (DAMGO, Sigma), 5-hydroxytryptamine (5-HT, Sigma), 5-carboxamidotryptamine (5-CT, Glaxo), (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene HBr (8-OH-DPAT, Research Biochemicals Inc.), {1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]-piperazine hydrobromide} (NAN-190, Research Biochemicals Inc.), spiperone (Research Biochemicals Inc.), ketanserin (Sigma), (3-tropanyl)-1-H-indole-3-carboxylic acid ester (ICS 205-930). Numerical data are presented as means ± S.E. of the means.

RESULTS

TWO TYPES OF CELLS

In a previous study in the rat NRM[26], primary and secondary cell types were characterized as follows. Primary cells had resting membrane potentials between -55 and -74 mV and did not fire action potentials spontaneously. The action potential duration measured at the threshold for activation was between 1-2.4 ms. Electrical stimulation caused a fast synaptic potential that was blocked by bicuculline and was therefore thought to be mediated by GABA. Opioids such as ME or DAMGO had no effect on the resting membrane potential of primary cells, but suppressed the amplitude of the GABA-mediated synaptic potential. Secondary cells were often spontaneously active. The membrane potential in this group of cells varried between -50 and -68 mV and the action potential duration was <1 ms. Electrical stimulation evoked a fast synaptic potential that was blocked by CNQX and AP-5 idicating that it was mediated by glutamate. Only secondary cells that were hyperpolarized by opioids were studied.

In this study intracelluiar recordings were made from a total of 102 cells in rat NRM and divided into two cell types (primary n=78, secondary n=24) according to the criteria

described above[26]. In some experiments, physiologically identified cells were filled with biocytin and characterized immunohistochemically. In these experiments, biocytin filled cells were recovered in 38 of 42 cases (91%). Biocytin-labeled cells were all found within the rostral portion of the B3 serotonin cell group of Dahlstrom and Fuxe[10], in the medial portion of the ventral brainstem. Most were found at the level of the trapezoid body.

Primary cells stained for 5-HT A total of 28 cells classified as primary cells physiologically were filled with biocytin, recovered and examined for 5-HT immunoreactivity. Of these, 26 cells (93%) were found to be immunoreactive for 5-HT. The other 2 cells were not stained for 5-HT. Figure 1 (upper panel) is an example of a primary cell that was immunoreactive for 5-HT. It appears that most cells identified physiologically as primary cells are serotonergic.

Secondary cells were not immunoreactive for 5-HT A total of 10 cells physiologically identified as secondary cells were filled with biocytin and recovered; 9 cells (90%) appeared to be unstained for 5-HT (Fig. 1, lower panel). One cell was immunoreactive for 5-HT.

5-HT ACTIONS ON PRIMARY CELLS

5-HT-induced hyperpolarization In 54 of 64 (84%) primary cells tested, perfusion of 5-HT caused a hyperpolarization (Fig. 2 A). The amplitude of the hyperpolarization induced by 5-HT (30 μM) was 11.4 ± 4.7 mV (range from 3 to 21 mV, n=54). The 5-HT-induced hyperpolarization was dependent on the concentration applied and had a threshold of <1 μM. The maximum hyperpolarization was obtained at about 30 μM and the EC_{50} was 3.5 μM (Fig. 2 B). All the experiments were conducted in the absence of any 5-HT reuptake blockers. Of 10 remaining primary cells, 3 cells (5%) showed no response to 5-HT and 7

cells (11%) were depolarized by 5-HT in the absence or presence of TTX (1μM). This depolarization was not characterized further.

The selective 5-HT_1 receptor agonist, 5-CT, produced a concentration dependent hyperpolarization in every cell tested (n=22). The threshold concentration was about 1 nM and maximum hyperpolarization was 12±3 mV at 100 nM. The concentration response curve for 5-CT had an EC_{50} of about 4 nM (n=6) and was shifted to the right in the presence of a selective 5-HT_{1A} receptor antagonist, NAN-190 (100 nM, n=5, Fig. 3). The EC_{50} for 5-CT in NAN-190 was 93 nM. The shift was parallel with the same maximum hyperpolarization as in control, suggesting a competitive antagonism. The estimated K_d for NAN-190 from this single shift was 4.5 nM.

The selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (1-100 nM), also caused a concentration dependent hyperpolarization with a maximum of 11±1mV (n=3) at 100 nM and an EC_{50} of 16 nM. Although the washout was not complete even after 1 hour, the 8-OH-DPAT induced hyperpolarization could be reversed by NAN-190 (10-100nM) or spiperone (1 μM). Ketanserin (1 μM) and ICS 205-930 (1 μM) did not affect the 8-OH-DPAT-induced hyperpolarization, indicating that 5-HT_2, 5-HT_{1C} and 5-HT_3 receptors were not involved.

Under voltage clamp, an outward current was induced by 5-HT or 5-CT at resting membrane potential associated with an increase in membrane conductance. The current reversed polarity at -96±6 mV (n=16, 2.5 mM extracellular potassium). As the potassium concentration in the perfusion solution was increased to 6.5 mM and 10.5 mM, the reversal potential shifted to -74±2 mV and -62±4 mV, respectively (n=9, Fig. 4). The 5-HT- or 5-CT-induced current exhibited characteristic inward rectification especially in higher extracellular potassium concentration as has been reported in dorsal raphe[40]. The shift in reversal potential had a slope of -55±2 mV/10 fold change in extracellular potassium concentration according to the Nernst equation, suggesting a relatively selective increase in

potassium conductance.

5-HT-mediated IPSP After a single electrical stimulation, several synaptic potentials were evoked, including a fast depolarizing synaptic potential that had a duration of about 200 ms, a slow inhibitory postsynaptic potential (IPSP, 1-2 s duration) and a slow EPSP that lasted several seconds. The following experiments were carried out in the presence of APV (30 μM), CNQX (10 μM) and bicuculline (10 μM) to block the fast synaptic potentials[26]. The α_1-adrenoceptor antagonist, prazosin (1 μM), was used to eliminate the slow EPSP, as has been reported in the dorsal raphe nucleus[25,41].

Under these conditions, IPSPs were found in 12 of 14 primary cells. When evoked from resting membrane potential, the amplitude of the IPSPs ranged from 4 to 13 mV, depending on the strength of stimulation. The duration of the IPSP was 1.8 ± 0.7 s (n=7). From the onset of the hyperpolarization to the return of the potential to control, the IPSP could be approximated by the sum of two exponentials with time constants of 170 ± 40 ms for the rising phase and 710 ± 180 ms for the decay component. The amplitude of the IPSP declined and then reversed polarity as the membrane potential was held at more negative potentials from resting. (Fig. 5 A). The reversal potential for the IPSP was -93 and -103 mV in two experiments.

Superfusion with 5-HT_{1A} receptor antagonists, NAN-190 (10 nM) or spiperone (1μM), completely blocked the IPSP in every cell tested (n=5, Fig. 5 B). It took at least 15 min to elicit the maximum effect of NAN-190 at 10 nM and the inhibition of the IPSP by both NAN-190 and spiperone did not wash out completely even after more than 1 hour.

5-HT ACTIONS ON SECONDARY CELLS

5-HT-induced hyperpolarization Out of a total of 24 secondary cells that were hyperpolarized by ME, 20 cells (83%) were also hyperpolarized by superfusion with 5-HT (10-30 μM, Fig. 6 A). The remaining 4 cells out of the 24 cells (17%) were not affected by 5-HT. The amplitude of the 5-HT-induced hyperpolarization was 6.5 ± 3.4 mV (n=20). The effective concentration range in these cells was similar to that in primary cells (1-30 μM).

In every tested secondary cell that was hyperpolarized by 5-HT, 5-CT caused a hyperpolarization as well (Fig. 6 B). The hyperpolarization induced by 5-CT (100-300 nM) was 6.5 ± 1 mV (n=4) in amplitude. Spontaneous firing was often eliminated by the hyperpolarization. The washout of 5-CT was much longer than that of 5-HT presumably because 5-CT is not a substrate for reuptake. 8-OH-DPAT (1-100 nM) also produced a concentration-dependent hyperpolarization (n=4) having a maximum of 6 mV at 100 nM. The hyperpolarization induced by both 5-CT and 8-OH-DPAT was blocked by NAN-190 (10-100 nM) or spiperone (1 μM), but not affected by ketanserin (1 μM) or ICS 205-930 (1 μM).

Under voltage clamp, an outward current was induced by 5-CT (Fig. 7 A) as well as by ME (Fig. 7 B). The 5-CT-induced current reversed polarity at the membrane potential of -97 ± 7 mV (n=5) and showed inward rectification similar to that induced by ME. In addition, when ME and 5-CT tested together there was no additional current. In one cell as the potassium concentration in the perfusing solution was increased to 6.5 mM and 10.5 mM, the reversal potentials for the 5-CT-induced current were shifted to -72 mV and -62 mV respectively, suggesting a relatively selective increase in conductance to potassium ions.

Although, fast EPSP's, blocked by CNQX (10 μM) and APV (30 μM), and slow

EPSP's, blocked by prazosin (100 nM) could be evoked in secondary cells, no slow IPSP's were observed (n=6).

DISCUSSION

GENERAL OBSERVATIONS

In the present study, 93% of primary cells recovered appeared to be stained for 5-HT. In contrast, only 10% of secondary cells recovered appeared to be immunoreactive for 5-HT. These findings suggest that most primary cells in our preparations contain 5-HT and that most secondary cells do not. Previous studies have shown that the inhibitory tone on primary cells is decreased by administration of opioids[26]. It would therefore appear that most 5-HT containing neurons in the NRM could be excited by opioids. The latter finding disagreed with previous reports made *in vivo* [2,9], possibly because of differences in the criteria used for identification of serotonergic neurons, or differences in the population of serotonergic neurons that were sampled. However, it is in agreement with numerous biochemical studies suggesting that opioids increase the activity of 5-HT containing neurons [7,33,34].

Twenty-seven out of the total of 38 cells recovered (71%) were immunoreactive for 5-HT. This percentage is higher than has previously been reported as the proportion of serotonergic cells in NRM[24,37], suggesting that we recorded preferentially from serotonergic neurons. This may be due to choice of recording site that was mainly in the ventromedial part of the NRM. This area is where the highest density of serotonergic cells was found in NRM[10,31]. Alternatively, the recording methods may have been preferentially successful with 5-HT containing primary cells.

5-HT-INDUCED HYPERPOLARIZATION

5-HT hyperpolarized most NRM cells, whether primary cells or secondary cells. In both primary and secondary cell groups, the hyperpolarization induced by 5-HT was mimicked by the 5-HT_1 receptor agonists, 5-CT and 8-OH-DPAT[8,28]. The 5-HT-receptor mediated hyperpolarization was antagonized by the 5-HT_{1A} receptor antagonists, NAN-190[16] and spiperone[8,28], but not affected by the 5-HT_2/5-HT_{1C} receptor antagonist ketanserin[8,28] or the 5-HT_3 receptor antagonist ICS 205-930[8,28]. These results indicate that the 5-HT-induced hyperpolarization is mediated by 5-HT_{1A} receptors in both primary and secondary cells. In voltage clamp, the 5-HT-induced outward current in both groups had a reversal potential close to potassium equilibrium potential. These results indicate that the 5-HT-mediated hyperpolarization in both primary and secondary cells is due to an increase in potassium conductance. This mechanism of the 5-HT action is similar to what has been characterized previously in dorsal raphe neurons[40]. In secondary cells, it is likely that 5-HT and opioids activate the same potassium channel, since when one agonist was applied in a concentration that caused a maximal effect, application of the second agonist caused no further increase.

Previous studies in vivo have shown that iontophoresis of 5-HT either inhibits all the identified raphe-spinal neurons in NRM[39] or inhibits one group of neurons and excites another group[11,12,38]. In the present study, 5-HT directly inhibited most NRM cells and only a small portion of cells (11%) were depolarized. The reason for this discrepancy is unclear. It could be that different populations of NRM cells were sampled especially since only the cells in the rostral portion of NRM were sampled in this study. Alternatively, differences between the *in vitro* and *in vivo* preparations could stem from the disruption of neuronal circuitry in the slice.

5-HT-MEDIATED IPSP

A 5-HT-mediated slow IPSP evoked by a single electrical stimulation was observed in primary cells. The IPSP had similar characteristics to the 5-HT_{1A} receptor-mediated IPSP described previously in dorsal raphe neurons[25], that is, it reversed polarity at the potassium equilibrium potential; was prolonged by the 5-HT reuptake blocker, cocaine; and was completely abolished by the 5-HT_{1A} receptor antagonist, NAN-190. These results indicate the existence of serotonergic inputs onto a subgroup of NRM cells (primary cells). Attempts to evoke IPSP's in secondary cells were not successful even though the cells were hyperpolarized by 5-HT. There are at least two explanations. First, the amplitude of hyperpolarization produced by 5-HT (30 μM) was in general smaller in secondary cells (6.5±4.3 mV) than in primary cells (11.4±4.7 mV), so that the IPSP amplitude in secondary cells might be expected to be small. In fact, in primary cells where exogenously applied 5-HT caused a hyperpolarization of less than 10 mV, it was difficult to evoke an IPSP. Secondly, since different groups of NRM neurons were shown to have different planar organization of dendritic fields and axon orientation[21], it is possible that there are few serotonergic inputs onto secondary cells in slices cut in the coronal plane.

PHYSIOLOGICAL SIGNIFICANCE OF 5-HT ACTIONS

It has been previously shown that opioids decrease GABA-mediated synaptic potentials onto primary cells[26]. This action suggests that opioids could activate primary cells by disinhibition. In the present study, most primary cells were found to be immunoreactive for 5-HT. Recording sites in this study were concentrated at the level of the trapezoid body. This portion of NRM is the region where most of the serotonergic neurons have been shown to project to the dorsal horn of the spinal cord[30]. Thus, it appears that this rostral portion of NRM is more likely to be involved in nociceptive modulation and that the activation (through disinhibition) of serotonergic primary cells in this region may contribute to the descending inhibition by opioids. Inhibition of these primary cells by 5-HT would be expected to cause hyperalgesia, whereas the inhibition of secondary cells by 5-HT as

well as by opioids, would produce analgesia. Unlike opioids, 5-HT inhibited both primary cells and secondary cells so that the effects of exogenously applied 5-HT in the NRM are expected to be complex. In fact, there are conflicting reports about the effect of systemic 5-HT_{1A} receptor agonists on nociceptive threshold[6]. It was also found recently[23] that systemic administration of selective 5-HT_{1A} receptor agonists attenuated opioid-induced antinociception in Wistar rats, but the antagonism was not complete compared with that by naloxone. 5-HT actions through 5-HT_{1A} receptors on both of the functionally different pain-modulating neurons in NRM (both primary and secondary neurons) could explain, at least in part, these observations.

It should be pointed out that previous studies *in vivo* have shown that there is a large population of non-serotonergic cells in NRM that is involved in pain modulation[4]. These cells could function as GABA-containing interneurons, GABA-containing projection neurons[18] or other groups of cells (including possible descending excitatory cells). As discussed above, the proportion of non-serotonergic cells found in this study were lower than one would expect from previous studies *in vivo*. NRM cells contain a variety of transmitters other than 5-HT and GABA, including enkephalin and substance P[32]. The function of these non-serotonergic cells in pain modulation is still poorly understood.

Based on the observation of a 5-HT-mediated IPSP in serotonergic primary cells, it seems that the 5-HT_{1A} receptor on serotonergic primary cells may function to regulate activity throught collateral inhibition as has been proposed previously in the dorsal raphe nucleus[35,36] . This is further supported by the finding that there are numerous serotonin-immunoreactive varicosities in the vicinity of serotonergic neurons in NRM[31]. Non-serotonergic secondary cells are also inhibited by activation of the 5-HT_{1A} receptor. Its functional significance is not known. Since it has been reported that there is a reciprocal relationship between the spontaneous firing of two types of putative nociceptive modulating

neurons in NRM of lightly anesthetized rats[3], one possibility is that the inhibition of these secondary cells may be synaptically mediated by the serotonergic primary cells. This synaptic interaction could partially account for the coordination of activity between serotonergic primary cells and non-serotonergic secondary cells. Opioids, in contrast, inhibit only secondary cells (consequently indirectly activating primary cells), so that opioids are efficient and consistent in production of antinociception.

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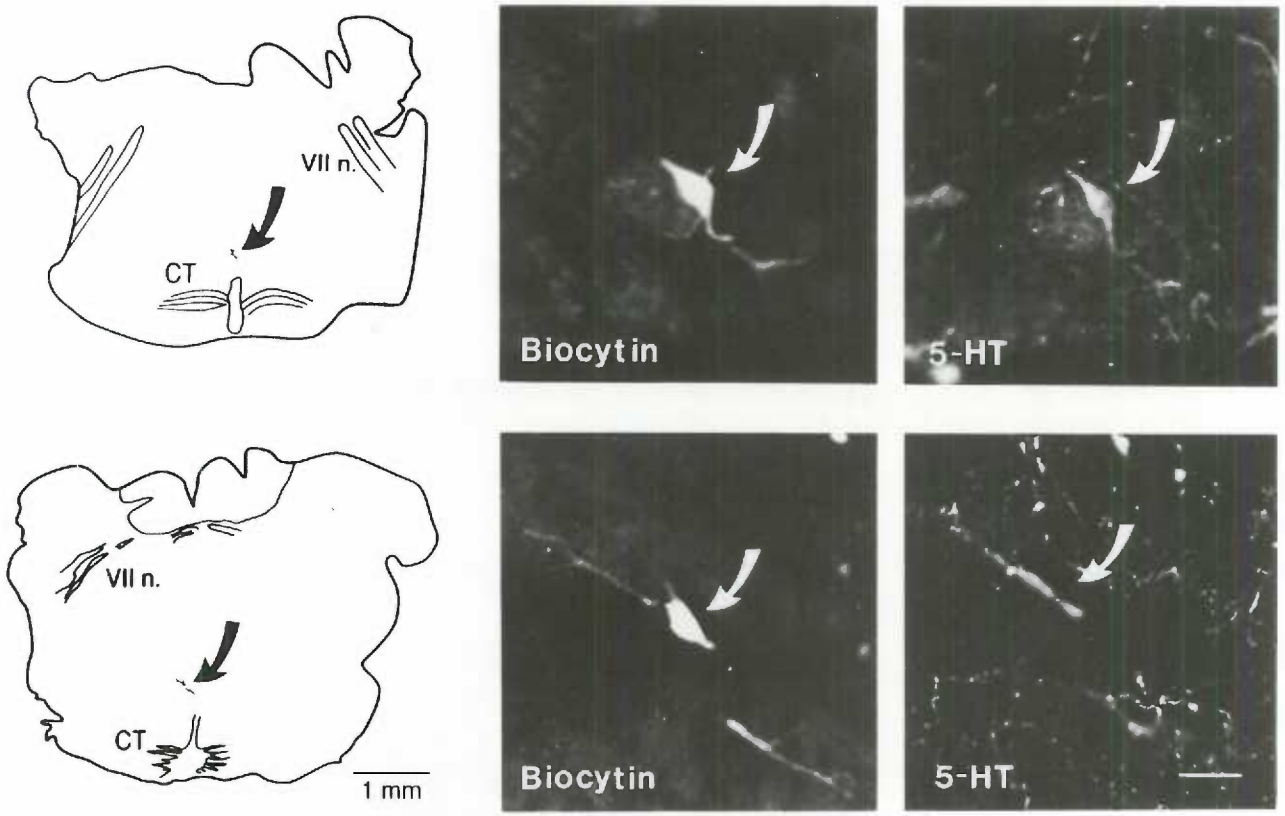

Fig. 1. Photographs illustrating a primary cell immunoreactive for 5-HT and a secondary cell not immunoreactive for 5-HT in nucleus raphe magnus. **Upper panel:** a primary cell immunoreactive for 5-HT. Left: camera lucida reconstruction of the recording site. Arrow points to the biocytin-labeled cell. Middle: biocytin-labeled cell, visualized using avidin conjugated with the blue fluorophore diethylaminocoumarin. Right: the same section, stained for 5-HT. Note that the cell is labeled for 5-HT. **Lower panel**: a secondary cell that is not immunoreactive for 5-HT. Left: camera lucida reconstruction of the recording site. Arrow points to the biocytin-labeled cell. Middle: biocytin-labeled cell, visualized using avidin conjugated with the blue fluorophore diethylaminocoumarin. Right: the same section, stained for 5-HT. Note that the cell is not labeled for 5-HT. CT=trapezoid body. VII n.= facial nerve. Bar=50 μm.

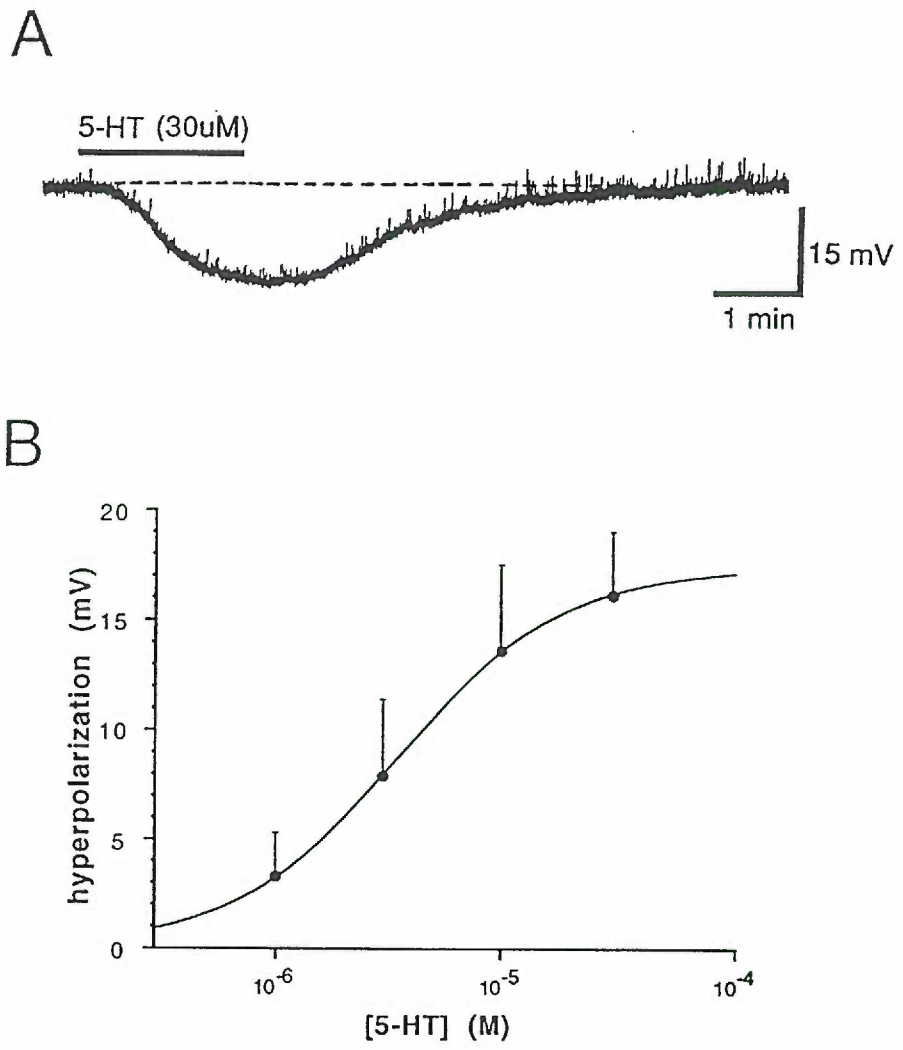

Fig. 2. Concentration-dependent hyperpolarization induced by superfusion of 5-HT (1-30 μM) in primary cells. A: a recording of the membrane potential from a primary cell. 5-HT (30μM) produced a hyperpolarization of 15 mV. B: Concentration response curve of the 5-HT-induced hyperpolarization summarized from 7 cells. Error bars are s.e.m.

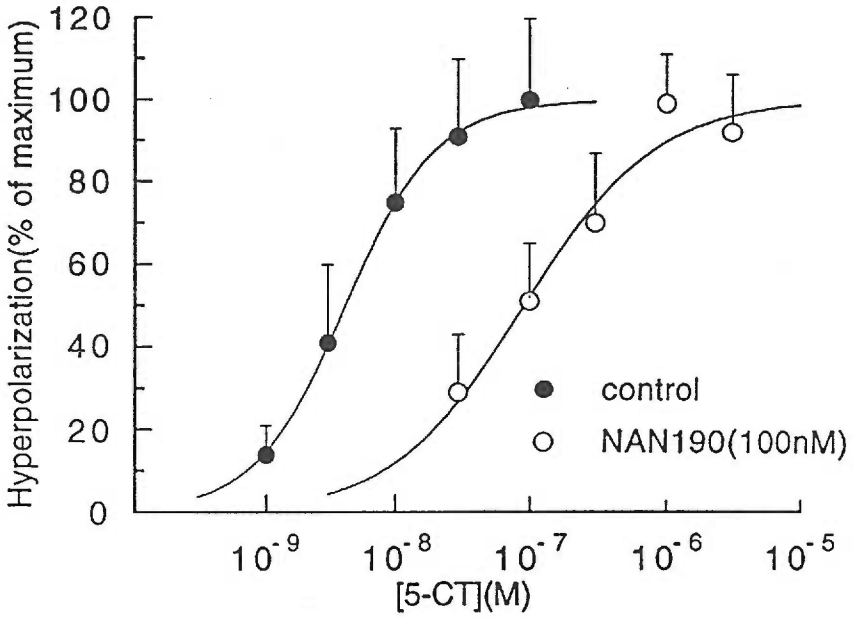

Fig. 3. Concentration dependence of the hyperpolarization induced by 5-CT in control (filled circles) and in the presence of NAN-190 (open circles). All data points are expressed as percentage of the maximum hyperpolarization (12 mV) induced by 5-HT at 30 μM. The EC_{50} for 5-CT was 4 nM in control (n=6) and 93 nM in NAN-190 (n=5). Error bars are sem.

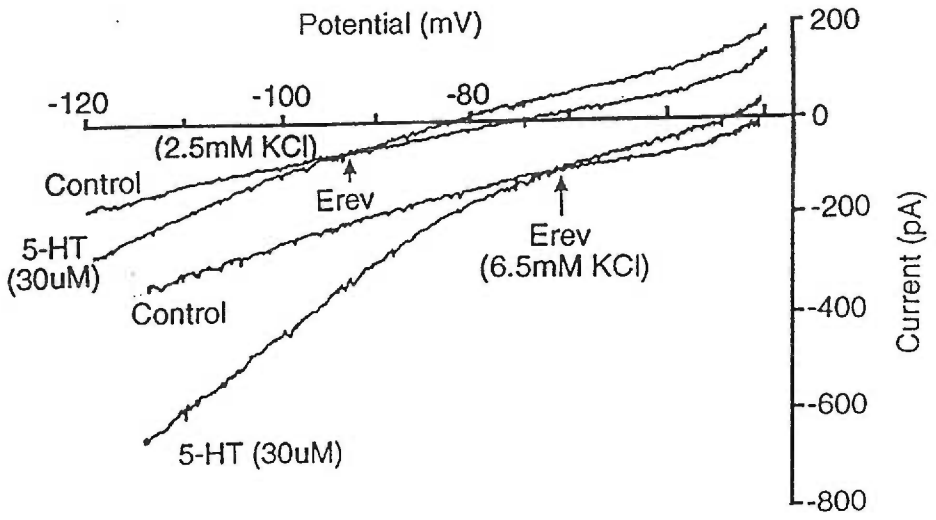

Fig. 4. Steady-state current-voltage plots in the presence and absence of 5-HT (30 μM) in two different extracellular potassium concentrations from a primary cell. The reversal potential (arrows) was shifted from -93 mV in 2.5 mM potassium to -71 mV in 10.5 mM potassium. Note the inward rectification of the 5-HT-induced current.

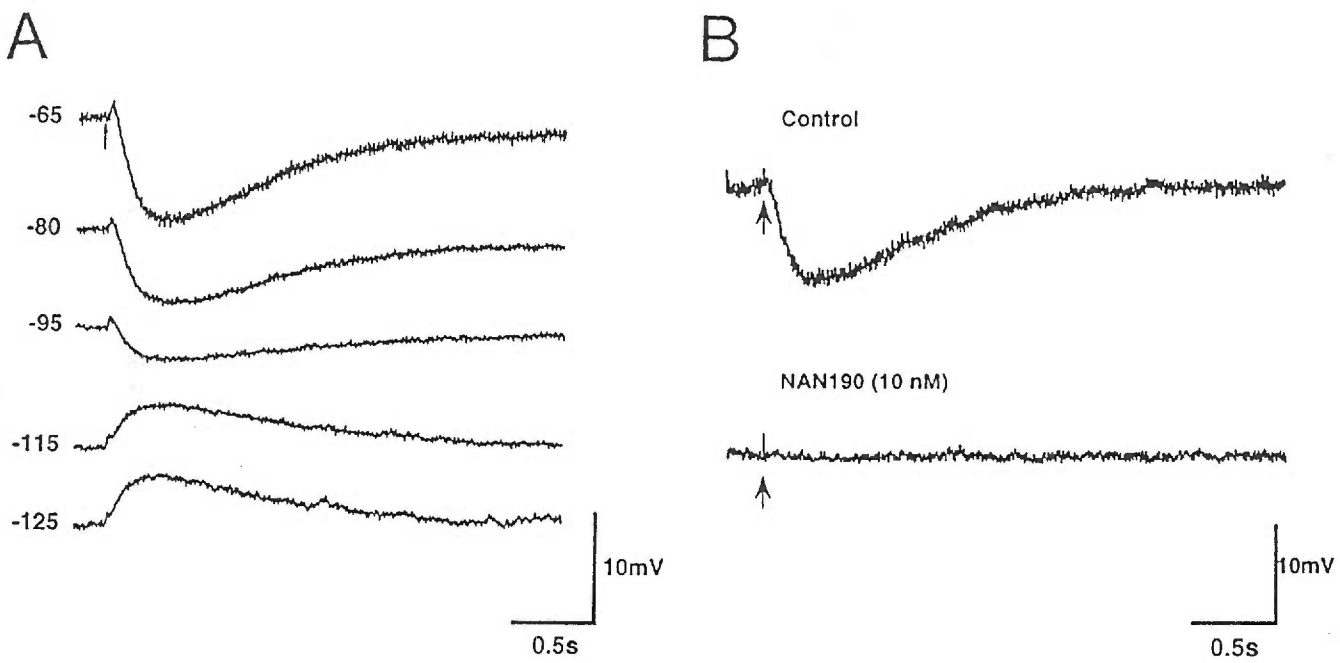

Fig. 5. 5-HT-mediated IPSP in two primary cells. A: An IPSP is evoked (arrow) while holding the membrane potential at the indicated potential. The IPSP reversed polarity at -103 mV. B: The IPSP evoked from another primary cell was completely abolished by NAN-190. All experiments were carried out in the presence of APV (30 μM), CNQX (10 μM), bicuculline (10 μM) and prazosin (1 μM).

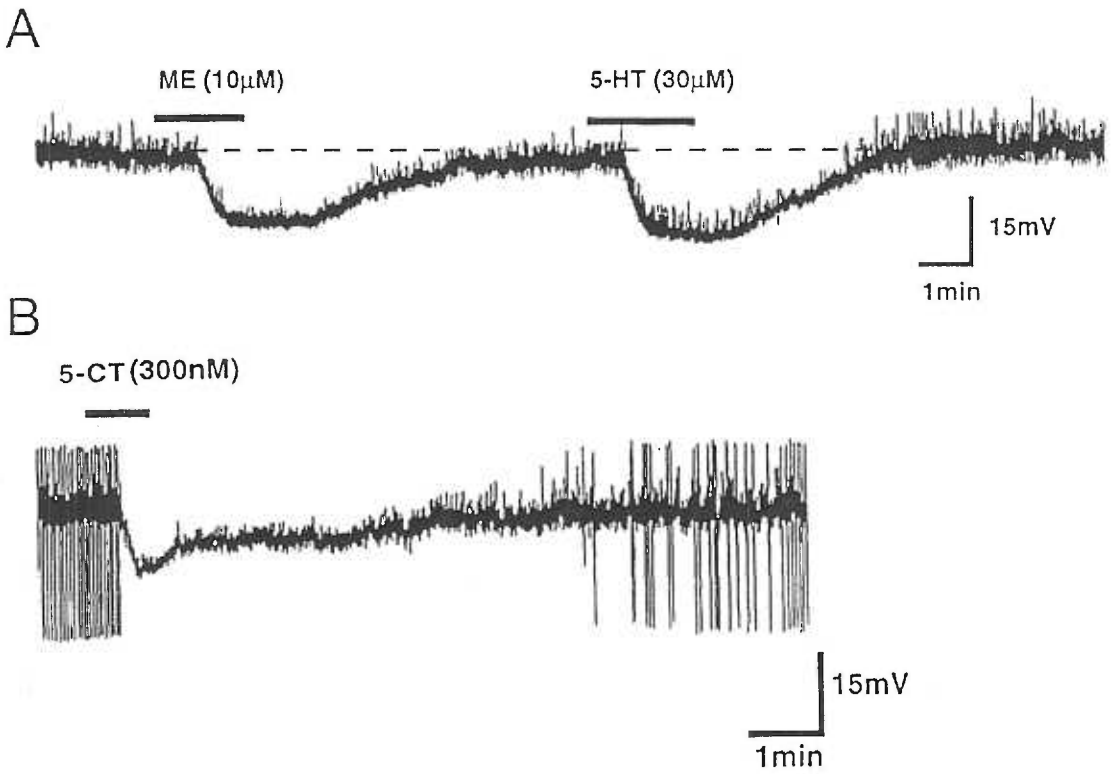

Fig. 6. Hyperpolarizations induced by 5-HT, 5-CT and methionine enkephalin (ME) in two secondary cells. A: a membrane potential recording from a secondary cell. Both 5-HT and ME produced a hyperpolarization in the same cell. B: A recording of membrane potential from another secondary cell that was spontaneously firing action potentials. 5-CT hyperpolarized the cell and eliminated the spontaneous firing.

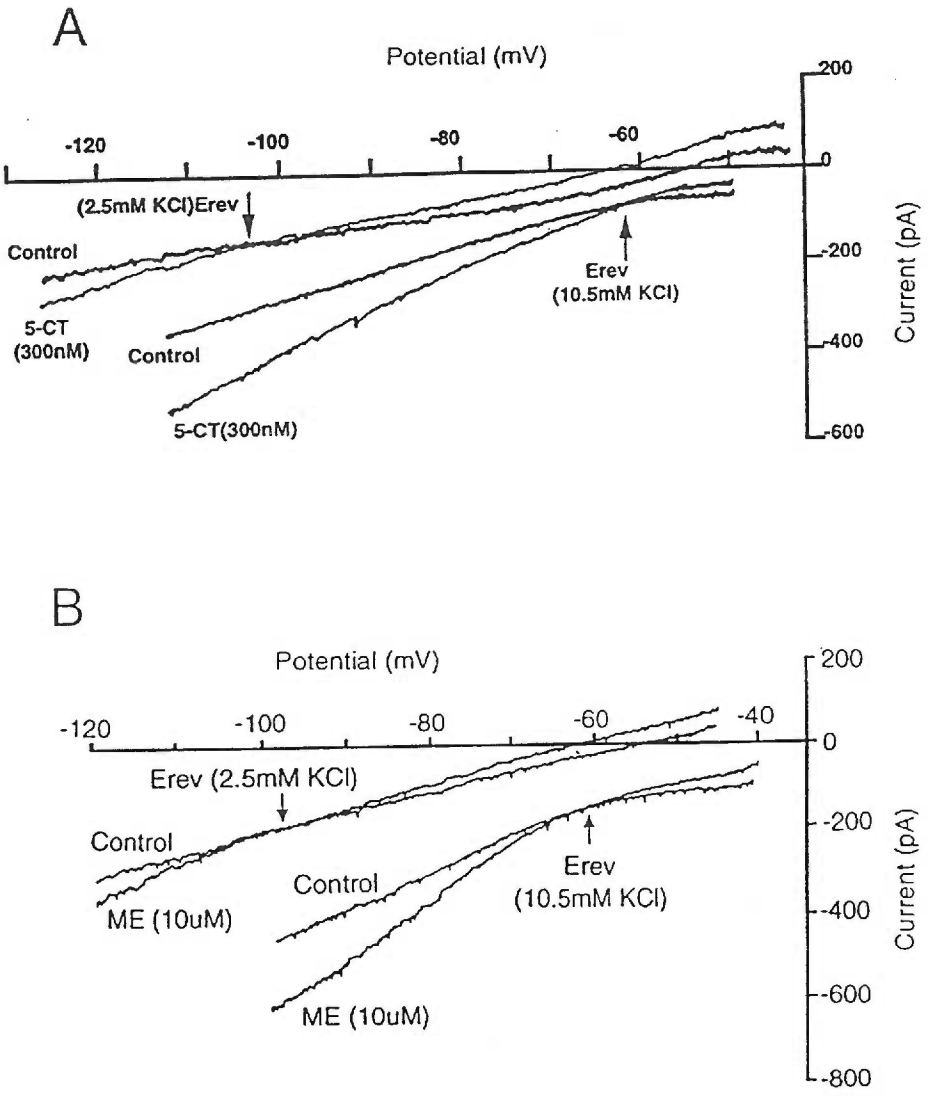

Fig. 7. Characterization of the currents induced by 5-CT and ME in secondary cells. A: Steady-state current-voltage plots in control and in the presence of 5-CT from a secondary cell. The reversal potential (arrows) was shifted from -103 mV to -62 mV when outside potassium concentration was increased from 2.5 mM to 10.5 mM. Note that the 5-CT-induced current rectified inwardly. B: Current-voltage plots in control and in the presence of ME from another secondary cell. Note the similar inward rectification in ME-induced current to that in 5-CT-induced current.

Manuscript 3:

Journal of Neuroscience (submitted)

Muscarine hyperpolarizes a subpopulation of neurons by activating an M_2 muscarinic receptor in rat nucleus raphe magnus *in vitro*.

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Pages: 9

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Z.Z. Pan and J.T. Williams*

Vollum Institute, Oregon Health Sciences University

3181 S.W. Sam Jackson Park Road, Portland, OR 97201

* Corresponding author: Tel (503)494-5465

FAX (503)494-6972

E. mail WilliamsJ@OHSU.EDU

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Abstract

It has previously been shown that the muscarinic cholinergic system in the nucleus raphe magnus (NRM) is involved in the modulation of nociception. In this study, we examined the direct actions of muscarine on the NRM neurons in a slice preparation. Muscarine (1-30μM) produced a dose-dependent hyperpolarization in a subpopulation of the NRM cells that contain 5-hydroxytryptamine (5-HT). In voltage clamp, the muscarine-induced outward current reversed polarity at the potassium equilibrium potential and was characterized by strong inward rectification. The reversal potential was dependent on external potassium concentration, suggesting that the hyperpolarization induced by muscarine was mediated through an increase in an inwardly rectifying potassium conductance. 5-HT also hyperpolarized these cells by increasing the same inwardly rectifying potassium conductance. The concentration-response curve for muscarine (EC_{50}=2.7 μM) was shifted in a parallel manner to the right by increasing concentrations of pirenzepine (300nM-3μM) and methoctramine (50nM- 200 nM). Schild analysis revealed that the equilibrium dissociation constant (K_d) was 230 nM for pirenzepine and was estimated to be less than 30 nM for methoctramine. These results indicate that the muscarinic receptor mediating the muscarine activation of the potassium conductance in these cells is of the M_2 subtype. The present results suggest an inhibitory cholinergic postsynaptic modulation on the activity of a subpopulation of serotonergic neurons which are involved in antinociceptive function in the NRM.

Key words: Muscarine, M_2 muscarinic receptor, inwardly rectifying potassium conductance, pirenzepine, methoctramine, nucleus raphe magnus

Introduction

Neurons in the nucleus raphe magnus (NRM) are involved in the descending modulation of nociceptive processing from supraspinal levels (Basbaum and Fields, 1984). Functionally, there are different groups of putative pain-modulating neurons in the NRM and modulation of the activity in these neurons by opioids and other neurotransmitters results in a significant change in pain threshold (Fields et al., 1983, 1991). Among those neurotransmitters is acetylcholine (ACh). Cholinergic (ACh-esterase-positive) cell bodies and terminals have been identified within the NRM in previous anatomical studies (Bowker et al., 1983; Jones and Beaudet, 1986; Sherriff et al., 1991). The NRM receives cholinergic afferents originating from the cells in the pedunculopontine tegmental nucleus (Rye et al., 1988). Behavioral studies have shown that local applications of both muscarinic receptor agonists and antagonists into the NRM cause antinociception (Brodie and Proudfit, 1986; Iwamoto, 1991). In previous *in vivo* studies, iontophoretic application of ACh has been reported to induce an inhibition or excitation in the spontaneous activity of different groups of the NRM cells (Behbehani, 1982; Wessendorf and Anderson, 1983; Willcockson et al., 1983; Hentall et al., 1993). The cellular mechanism underlying the actions of these cholinergic agents on the NRM neurons is not known. Nor are the muscarinic receptor subtypes that mediate the cholinergic actions on the NRM neurons.

The actions of ACh acting at muscarinic receptors in the CNS include a depolarization resulting from a reduction in a potassium conductance (Brown and Adams, 1980; Madison et al., 1987; Uchimura and North, 1990) and a hyperpolarization as a result of an increase in a potassium conductance (Egan and North, 1986; McCormick and Prince, 1987; McCormick and Pape, 1988; Gerber et al., 1991). Muscarinic cholinergic receptors consist of a heterogeneous family of five different subtypes (m_1 through m_5) that have been cloned from rats (Kubo et al., 1986; Bonner et al., 1987, 1988). The m_1, m_2 and m_3 receptors correspond most closely to the pharmacologically defined M_1, M_2 and M_3

receptors (Kubo et al., 1986; Brann et al., 1988; Maeda et al., 1988; Buckley et al., 1989). The m_1, m_3 and m_5 receptors are functionally coupled primarily to the stimulation of inositolphosphate metabolism through a pertussis toxin-insensitive G protein while the m_2 and m_4 receptors are associated mainly with an inhibition of cAMP production through a pertussis toxin-sensitive G protein (Bonner et al., 1988; Fukuda et al., 1988; Peralta et al., 1988; Wess et al., 1990; Lai et al., 1991). In rat brainstem, the presence of all five receptor subtypes or their mRNAs has been reported (Buckley et al., 1988; Ehlert and Tran, 1990; Levey et al., 1991; Li et al., 1991; Zubieta and Frey, 1993).

In this study, we examined the direct actions of muscarine on the neurons in the NRM in a slice preparation and then identified the ionic mechanism and the receptor subtype involved in the muscarine-induced hyperpolarization observed in majority of the neurons in the NRM. Preliminary results of this study have been presented as an abstract (Pan and Williams, 1991).

Methods

Intracellular recordings were made from NRM cells in brain slices from adult Wistar rats. The methods employed were similar to those published previously (Pan et al., 1990). Briefly, brain slices (300 μm thick) were cut in a vibratome in cold (4 °C) physiological saline. Two or three coronal slices were taken from the level of the facial nerve in each brain. A single slice was submerged in a tissue bath through which flowed physiological saline (1.5 ml/min) at 37 °C. The content of physiological saline solution was (mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; CaCl, 2.4; glucose, 11; $NaHCO_3$, 25; gassed with 95% O_2, 5% CO_2 at 37 °C.

The NRM was recognized in the slice as a triangular area in the midline just above the

pyramidal tracts. Neurons were penetrated with glass microelectrodes filled with potassium chloride (2 M) having a resistance of 40-70 MΩ. Membrane currents were recorded with a single-electrode voltage-clamp amplifier (Axoclamp 2A) using switching frequencies between 3 and 6 KHz. The setting time of the clamp following a 10 mV step was typically 3-5 ms. Steady-state current-voltage (I-V) plots were constructed directly on an x/y plotter using a slow depolarizing ramp potential. The speed of the ramp (1 mV/s) was sufficiently slow to give the same current as that measured at the termination of a 2 second voltage step.

Drugs were applied by superfusion. The following drugs and salts were used: muscarine (Sigma), carbachol (Sigma), pirenzepine (PZP, Research Biochemical Inc.), methoctramine (Research Biochemical Inc.), 5-hydroxytryptamine (5-HT, Sigma), 5-carboxamidotryptamine (5-CT, Glaxo), $BaCl_2$ (Sigma), tetrodotoxin (TTX, Sigma). Dose response data were fit by logistic equation and the EC_{50} was then determined. Schild analysis was carried out by determining the dose ratios at the EC_{50} values in the absence and presence of the antagonist (Schild, 1949). Numerical data are presented as means $\pm$ S.E. of the means and compared using an unpaired, two-tailed Student's t test.

Results

Muscarinic agonists were tested on a total of 148 cells recorded intracellularly in the NRM. The resting membrane potential was between -50 and -78 mV with a resting slope conductance of 6.9 $\pm$ 0.6 nS (range 3 to 13 nS, n=27) measured between -50 to -60 mV. The majority of the cells (94%) were not spontaneously active and the duration of the evoked action potential measured at the threshold for activation ranged from 0.5 to 2.6 ms.

Muscarine hyperpolarized the majority of the cells tested in the NRM

Out of a total of 148 cells tested, muscarine caused a dose dependent hyperpolarization in 73 cells (49%, Fig.1A). This muscarine-induced hyperpolarization had a threshold at 1 μM and a maximum hyperpolarization of 9.8 ± 1.1 mV (n=8) at 30μM. The EC_{50} was 2.7 μM (Fig.1B). Carbachol, a muscarinic receptor agonist, also hyperpolarized these cells in a dose-dependent manner over a similar concentration range (1 to 30 μM, Fig.1B), inducing a maximum hyperpolarization of 9.4 ± 1.1 mV (n=7) at 30 μM and having an EC_{50} of 3.8 μM.

In 21 of 148 cells (14%), muscarine (10 μM) induced a depolarization of 4.8 ± 0.6 mV (n=21) which often resulted in the firing of repetitive action potentials. The remaining 54 cells (37%) did not respond to the above muscarinic agonists. TTX (1 μM) did not change the response of these cells to the agonists.

Muscarine hyperpolarized the cell by increasing an inwardly rectifying potassium conductance

In voltage clamp, muscarine produced an outward current at resting membrane potential associated with an increase in membrane conductance (n=24, Fig.2A). At more negative potentials than the resting potential, the muscarine-induced current declined in amplitude and reversed polarity. The averaged reversal potential of the muscarine-induced current was -103 ± 2 mV (n=17) in normal concentration of external potassium (2.5 mM). When the potassium concentration in the perfusing solution was increased to 6.5 mM and 10.5 mM, the reversal potential was shifted to -78 ± 2 mV (n=11) and -66 ± 2 mV (n=7), respectively (Fig.2B), giving a Nernst slope of -59.5 (Fig. 2C). This suggested a primary involvement of a potassium conductance.

The muscarine-induced current exhibited inward rectification that was more obvious in higher concentrations of external potassium (Fig.3A,C). In 6.5 mM external potassium, the slope conductance of the muscarine-induced current measured between -50 and -60 mV was 1.0 ± 0.2 nS and 5.2 ± 1.0 nS between -100 and -110 mV (n=10). Barium (30 μM) blocked the inwardly rectifying part of the muscarine current leaving a small, linear current (Fig.3B,C).

Muscarine and 5-HT hyperpolarized the cell by increasing the same inwardly rectifying potassium conductance

The effect of muscarine was tested in the cells that were hyperpolarized by 5-HT (10 or 30 μM). Out of 102 cells which were hyperpolarized by 5-HT, 58 cells (57%) were hyperpolarized by muscarine (Fig.4A). The hyperpolarizations induced by muscarine and by 5-HT were not additive. The membrane potential of other 5-HT responding cells was either not affected (n=41, 40%) or depolarized (n=3, 3%) by muscarine (10 or 30 μM).

The currents induced by muscarine and 5-HT in voltage clamp had the same inward rectification and barium sensitivity. The slope conductance of the 5-HT-induced current was 1.2 ± 0.1 nS between -50 and -60 mV and 8.4 ± 0.8 nS between -100 and -110 mV. $BaCl_2$ (30μM) abolished the inward rectification of the current induced by the 5-HT_1 receptor agonist 5-CT (300nM) (Fig.4C). Muscarine (30 μM) caused no more current in cells that were already treated with 5-HT (30 μM) or 5-CT (300nM) (n=4, Fig.4B). These results suggest that the same inwardly rectifying potassium conductance is involved in the muscarine- and 5-HT-induced hyperpolarization in these NRM cells.

Muscarine-induced hyperpolarization is mediated through an M_2 receptor subtype

To identify the receptor subtype involved in the muscarine action, the concentration-response relationship for muscarine was constructed in the absence and presence of different concentrations of the muscarinic receptor antagonists pirenzepine (PZP) and methoctramine.

The control dose-response curve for muscarine (EC_{50}= 2.7 μM) was shifted to the right by increasing concentrations of PZP (Fig.5A). These shifts were parallel and the same maximum effect was maintained, indicating a competitive antagonism. The EC_{50} for muscarine was 6.0 μM, 15.4 μM and 44.3 μM in 300 nM (n= 4), 1μM (n= 4) and 3 μM PZP (n= 3), respectively. The K_d for PZP for the receptor subtype mediating the muscarine response was estimated using Schild analysis. The resulting Schild plot (Fig.5B) was linear and the best-fit line through the data points had a slope of 1.05 that is not significantly different from unity. The pK_B values were calculated from individual concentration of PZP and corresponding dose ratios according to the equation: $pK_{Bi} = \log(DR-1)_i - \log[PZP]_i$. The mean pK_B calculated from each pK_{Bi} (n=11) was 6.64 ± 0.07 (95% confidence limits 6.49-6.79) and the Kd for PZP was 230 nM (162-324 nM). Regression of calculated pK_B estimates on the corresponding concentrations of PZP is shown in Fig.5C. No significant regression was obtained (t=0.4, d.f.=9), indicating that the estimated pK_B values (thus the K_d for PZP) were independent of the antagonist concentration.

The dose response curve for muscarine was also shifted to the right with the same maximum effect by increasing concentrations of methoctramine (Fig.6A). The EC_{50} for muscarine was 10.7 μM and 23.1 μM in 50 nM (n=5) and 100 nM (n=4) methoctramine, respectively. In 200 nM methoctramine (n=3), the EC_{50} was 83.9 μM, 31-fold larger than that in control. The Schild plot shown in Fig.6B had a best-fit line (x-intercept = -7.56)

with a slope of 1.67. This value was significantly different from unity, possibly indicating nonequilibrium steady-states in drug-receptor interactions at the lower antagonist concentrations (see discussion). The K_d value calculated from the dose ratio (31-fold) at 200 nM methoctramine was then estimated to be 7 nM, according to the equation: K_d=[antagonist]/(DR-1) (Kachur et al., 1990).

Discussion

The muscarinic receptor subtype involved

The coupling of muscarinic receptor activation to a potassium conductance increase in the CNS was first reported by Egan and North (1986) in rat parabrachial neurons. Involvement of an M_2 (previous definition) subtype was suggested based on the lower PZP affinity for the receptor (K_d=600 nM) versus the higher PZP affinity sites (M_1) (Hammer et al., 1980). Further development of muscarinic receptor antagonists has resulted in the current recognition of three pharmacologically defined subtypes with the M_2 further divided into M_2 (previously defined cardiac M_2) and M_3 (previous glandular M_2) (Bonner, 1989). In addition, five different muscarinic receptors have been cloned and radioligand binding studies have demonstrated that none of the antagonists has 5-fold or higher affinity for one subtype over another (Buckley et al., 1989). In the present study, PZP and methoctramine were used to determine the receptor subtype involved because of their higher ability to discriminate by affinity the M_1 and M_2 subtypes, respectively, from other subtypes (Buckley et al., 1989).

Previously in both radioligand binding studies (Akiba et al., 1988; Lai et al., 1988; Buckley et al., 1989; Lazareno and Roberts, 1989; Mei et al., 1989; Dorje et al., 1991) and

functional studies (Lai et al., 1988; Lazareno and Roberts, 1989; McKinney et al., 1989a, 1989b; Kachur et al., 1990; Sugita et al., 1991), PZP has been shown to have the following rank order of potency: M_1 (K_d range 3 - 33 nM) > m_4 (37 -120 nM, binding studies) > m_5 (89 - 170 nM, binding studies) ≥ M_3 (102 - 186 nM) > M_2 (219 - 906 nM). Our results indicate that PZP had a K_d of 230 nM for the receptor mediating the muscarine response, suggesting that the receptor is a non-M_1 subtype and the PZP K_d falls within the previously reported K_d range for the M_2 subtype.

The rank order of potency for methoctramine has been shown as follows: M_2 (K_d range 4 - 47 nM) > M_1 (16 - 50 nM) > m_4 (32 - 40 nM) > m_5 (57 - 135 nM) > M_3 (118 - 776 nM) from both binding studies (Buckley et al., 1989; Lazareno and Roberts, 1989; Wess et al., 1990; Dorje et al., 1991) and functional studies (Melchiorre et al., 1987; Lazareno and Roberts, 1989; McKinney et al., 1989b; Kachur et al., 1990; Sugita et al., 1991). Among the selective M_2 antagonists, methoctramine is comparatively the best in discriminating M_2 from the other three subtypes with the M_1 subtype excluded (Bonner, 1989). In the present study, no reliable K_d value for methoctramine could be obtained as the slope of the Schild plot was significantly greater than unity. This most likely resulted from nonequilibrium steady-states in the drug-receptor interactions at the lower antagonist concentrations, due to an inadequate period of time (20 min in this study) for equilibrium to be achieved. This would result in an underestimate of the dose ratio at the lower antagonist concentrations and a steeper Schild regression slope. If this is the case, the equilibrium conditions could be obtained by increasing the interaction time at lower concentrations of the antagonist (practically difficult in this study). The K_d estimate with a slope of unity would then be smaller than the value indicated by the x-intercept of a fitted line with the slope greater than unity (Kenakin, 1987). In our case, the actual pK_B value was probably less than -7.56 as indicated by the x-intercept of best-fit line through the data points (thus K_d < 28 nM). As an estimate, the K_d calculated from the dose ratio (31-fold) at 200 nM methoctramine was 7 nM. Therefore, the K_d for methoctramine appears to be <30 nM, which is consistent with the previously described methoctramine K_d range for the M_2

subtype.

Taken together, it seems reasonable to conclude that the muscarinic receptor mediating the hyperpolarization in these NRM cells is of the M_2 subtype. This is in agreement with previous anatomical studies where a relatively high density of M_2, and low density of M_1 and M_3 receptors (Buckley et al., 1988; Ehlert and Tran, 1990; Levey et al., 1991; Li et al., 1991; Zubieta and Frey, 1993) were found in rat brainstem structures including the NRM (Quirion et al., 1989). Only low level of m_4 and m_5 receptor proteins were detected in the rat brainstem area (Levey et al., 1991; Yasuda et al., 1993).

The inwardly rectifying potassium conductance

We have shown that the muscarine-induced hyperpolarization in these NRM neurons was mediated through an increase in a barium-sensitive, inwardly rectifying potassium conductance. The muscarinic receptor activation of the potassium conductance originally reported in the CNS parabrachial neurons (Egan and North, 1986) was linearly dependent on the membrane potential. Recently, Gerber et al. (1991) characterized an inwardly rectifying potassium conductance activated by muscarinic agonists in rat pontine reticular formation neurons and the involvement of a non-M_1 subtype was suggested. The potassium conductance in that study had similar characteristics in both the membrane potential dependence (inward rectification) and barium sensitivity to the ones described here. We have also shown that both muscarine and 5-HT hyperpolarized a group of the NRM cells through an increase in a similar inwardly rectifying potassium conductance and their effects were not additive. This suggests that the 5-HT_{1A} receptor (Pan et al., 1993) and the M_2 muscarinic receptor are probably coupled to the same inwardly rectifying potassium conductance in these neurons.

Functional considerations

The most commonly observed effect of muscarine on the NRM cells was a hyperpolarization (78% of muscarine responses). In a previous study , two groups of cells in the NRM have been described: the primary cell and the secondary cell (Pan et al., 1990). Primary cells have action potential duration of >1ms and are predominantly 5-HT-containing (93%) (Pan et al., 1993); Opioids do not affect the resting membrane potential but inhibit the GABA synaptic potential in primary cells. Secondary cells have shorter action potential duration (<1ms) and are hyperpolarized by opioids. In the present study, all primary cells (n=41) were hyperpolarized by muscarine and no depolarization was observed. These results are consistent with some previous *in vivo* studies where iontophoretic application of ACh into the NRM inhibited the spontaneous activity in the majority of the cells (Wessendorf and Anderson, 1983; Hentall et al., 1993). In addition, a more consistent inhibition was found specifically in raphe-spinal serotonergic cells (Wessendorf and Anderson, 1983). Other studies, however, have reported excitation of most cells in the NRM in response to iontophoretic ACh (Behbehani, 1982; Willcockson et al., 1983). This discrepancy may largely be attributed to the use of ACh that can act on both muscarinic and nicotinic receptors. Both receptor types are thought to be involved in modulation of the cellular activity and of antinociceptive function of the NRM (Iwamoto, 1991).

Generally, the activation of the cells, particularly those raphe-spinal projection cells, in the NRM produces antinociception through descending inhibition (Basbaum and Fields, 1984). A recent behavioral study has shown that local injection of methoctramine into the NRM produced a strong antinociceptive response in rats (Iwamoto, 1991). This implies the possibility of a tonic inhibitory muscarinic modulation of the NRM neurons responsible for the descending inhibition. In an earlier report, local application of the muscarinic receptor agonist carbachol into the NRM also induced antinociception. However, this effect did not appear to be mediated by serotonergic neurons in the NRM (Brodie and

Proudfit, 1986). The present results implicate an inhibitory cholinergic postsynaptic modulation on some of the serotonergic neurons in the NRM. These same neurons could presumably be activated by opioids through GABA-mediated disinhibition and may contribute to the descending modulation of sensory inputs (Pan et al., 1990).

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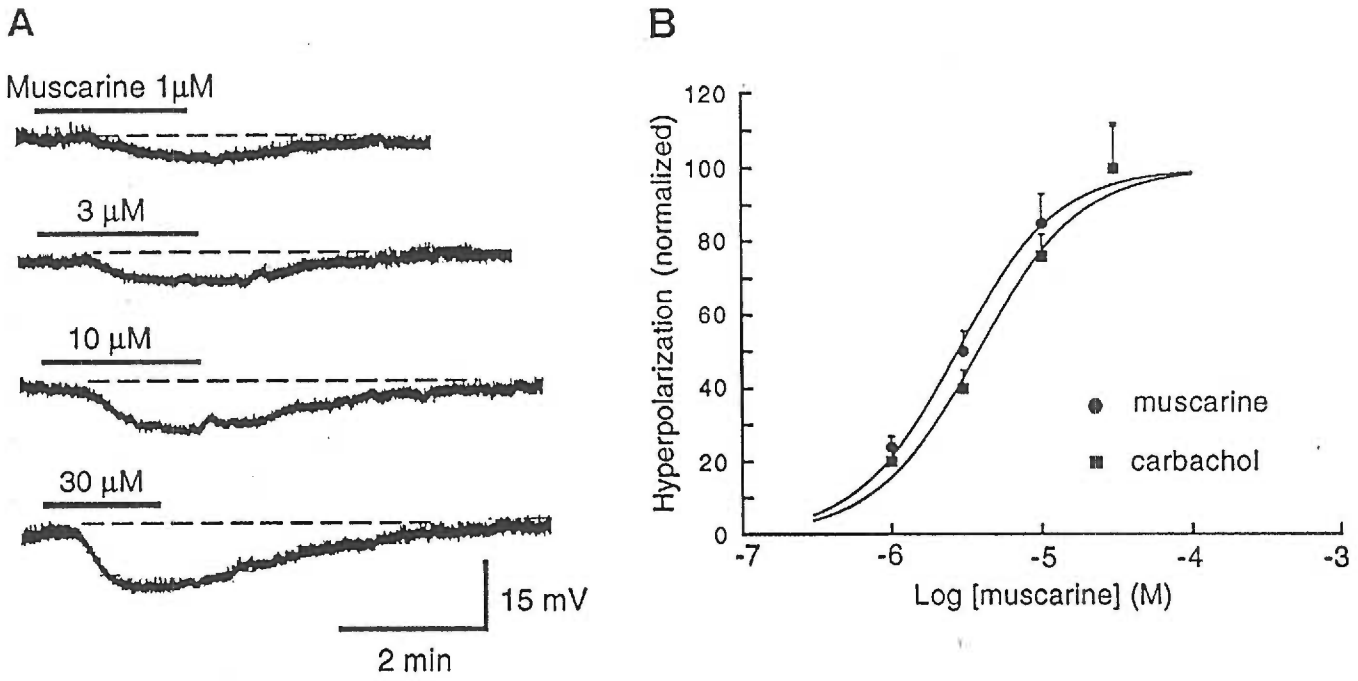

Fig.1 Hyperpolarization induced by muscarinic receptor agonists in neurons in the nucleus raphe magnus (NRM). A, Membrane potential recordings of a NRM cell that was hyperpolarized by increasing concentrations of muscarine. B, Dose-response curves for the hyperpolarization induced by muscarine (EC_{50}=2.7 μM, n=8) and by carbachol (EC_{50}=3.8 μM, n=7). Data points are expressed as a percentage of the maximum hyperpolarization by 30 μM muscarine (9.8±1.1 mV) and by 30 μM carbachol (9.4±1.1 mV), respectively. Error bars are S.E. of the mean.

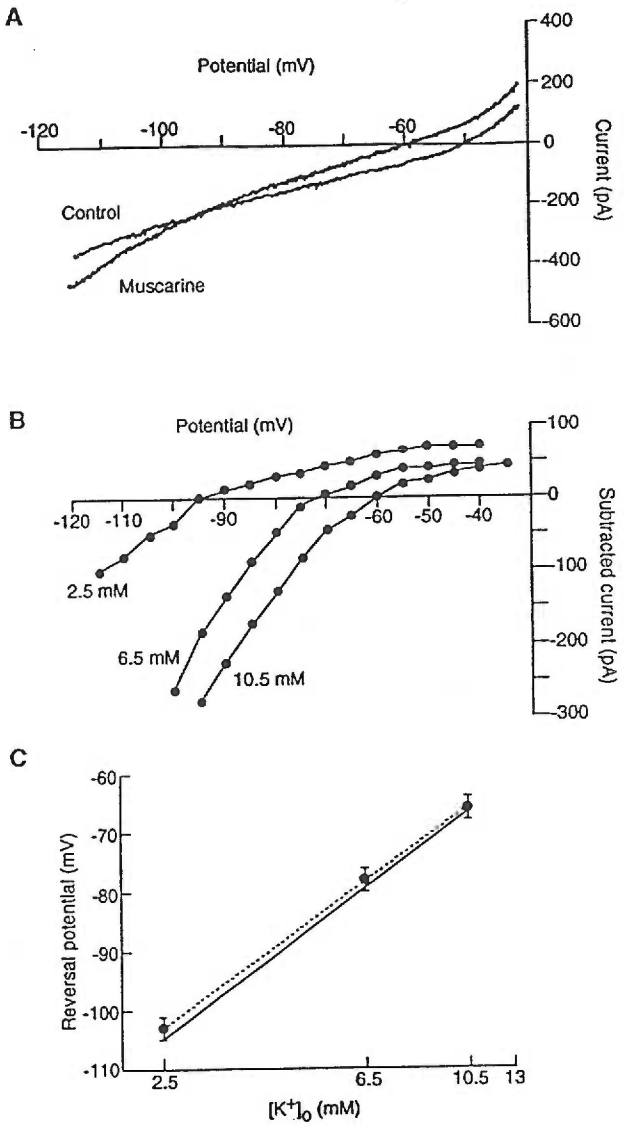

Fig.2 Muscarine-induced hyperpolarization is mediated through an increase in an inwardly rectifying potassium conductance. A, Steady-state current-voltage plots in the absence and presence of muscarine (10 μM). The reversal potential is -94 mV. B, The current-voltage plots of the muscarine-induced current in three different external potassium concentrations from the same cell as in A. The muscarine-induced currents were determined by manual subtraction of the current in control from that in muscarine at 5 mV intervals. C, Dependence of the reversal potential of the muscarine current on the external potassium concentrations. The dotted line is the fit to the data points (filled circles, means in seven to seventeen experiments, slope = -59.5). The solid line was determined by the Nernst equation where the internal potassium concentration was fixed at 135 mM.

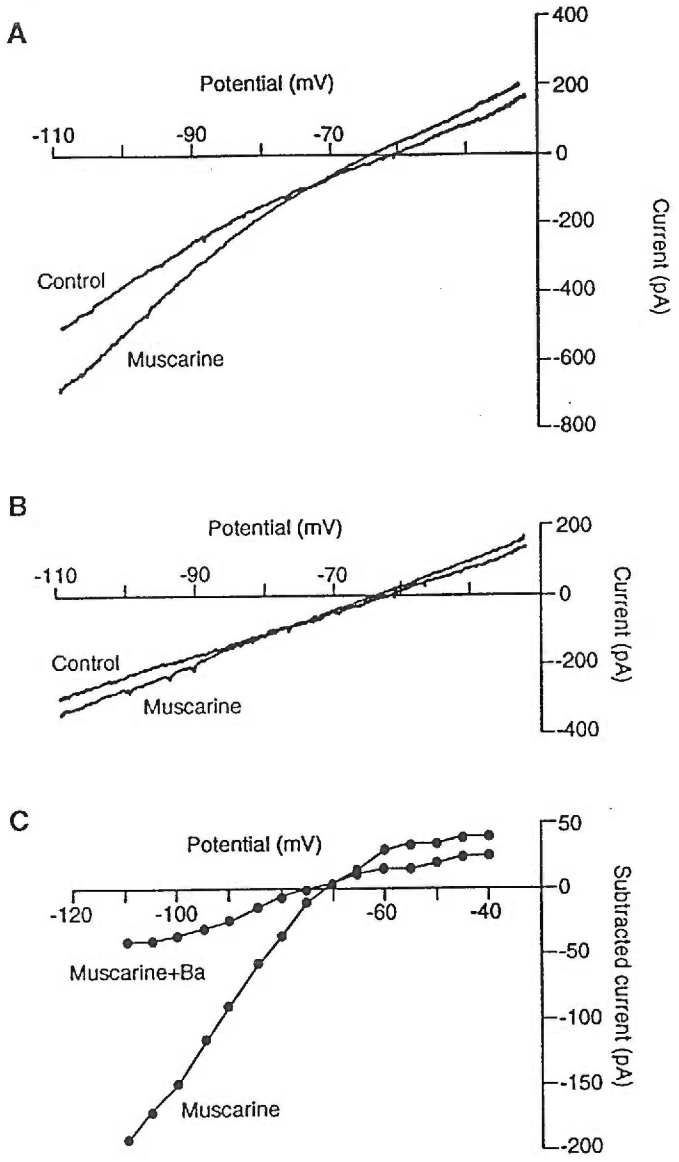

Fig.3 Inward rectification of the muscarine current. The experiment was carried out in 6.5 mM external potassium concentration. A, Steady-state current-voltage plots in control and in muscarine (10 μM). The muscarine current rectifies inwardly. B, Current-voltage plots in control and in 10μM muscarine after addition of $BaCl_2$ (30 μM), from the same cell as in A. C, Subtracted muscarine currents determined from A and B, showing that the slope conductance of the muscarine current increased as the membrane potential was shifted to more negative potentials and that barium blocked the inward rectification of the muscarine current.

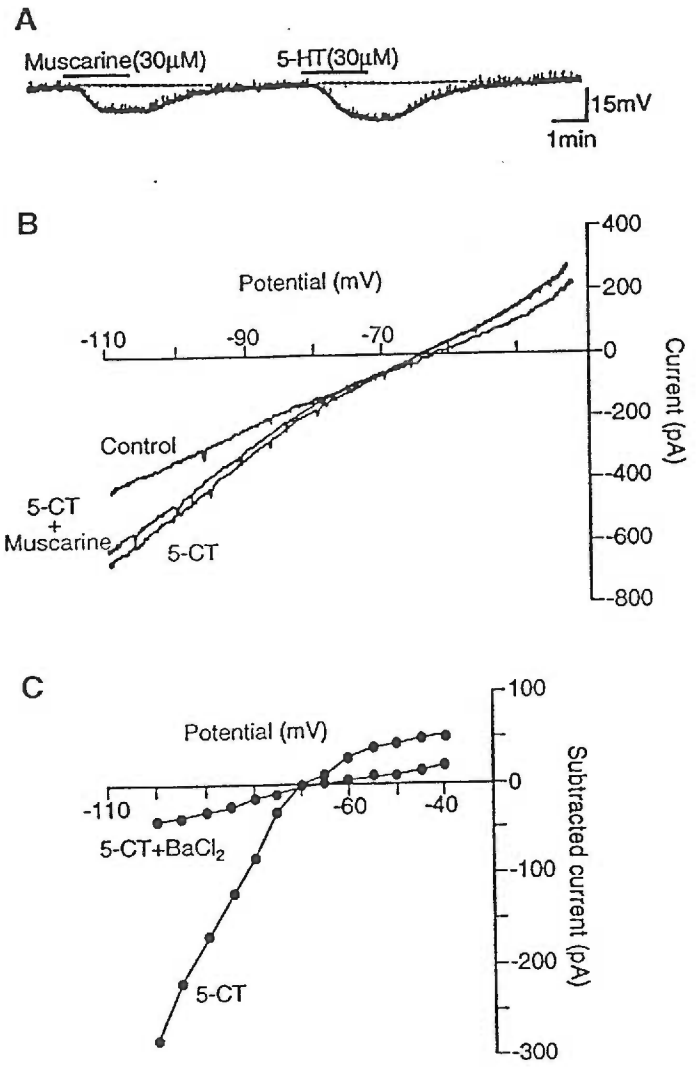

Fig.4 Muscarine and 5-HT hyperpolarize the cell by increasing the same inwardly rectifying potassium conductance. A, A membrane potential recording of a cell that was hyperpolarized by both muscarine and 5-HT. B, Steady-state current-voltage plots in control, in the presence of 5-CT (300nM), a selective $5HT_1$ receptor agonist and in the presence of 5-CT (300nM) plus muscarine (10 μM). In the presence of 5-CT, addition of muscarine caused no more current. C, Current-voltage plots of the subtracted 5-CT currents in control and in $BaCl_2$ (30μM) from another cell. Note the similarities in inward rectification and barium sensitivity of the 5-CT current to the muscarine current shown in fig.3C.

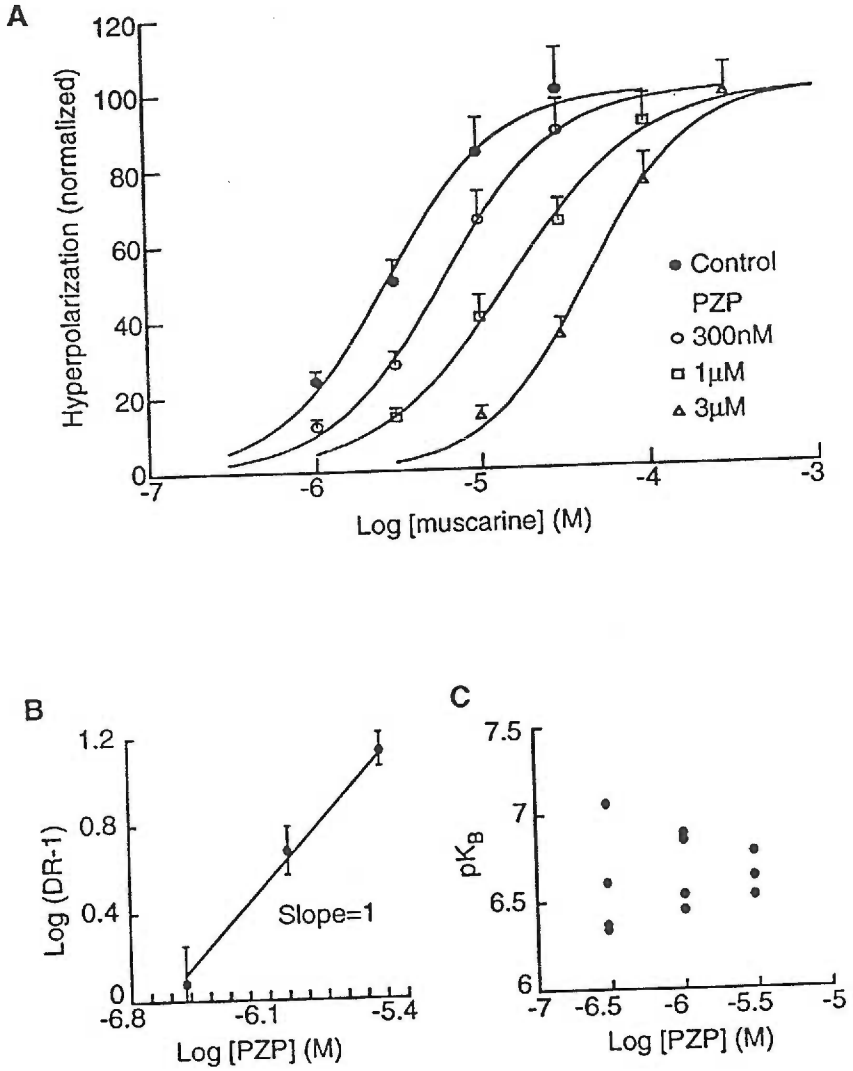

Fig.5 Competitive antagonism of the muscarine-induced hyperpolarization by increasing concentrations of pirenzepine (PZP). A, Dose-response curves for muscarine in control (●, EC_{50}=2.7 μM, n=8) and in the presence of 300nM (○, EC_{50}=6.0 μM, n=4), 1μM(□, EC_{50}=15.4 μM, n=4) and 3 μM PZP (Δ, EC_{50}=44.3 μM, n=3). Data points are presented as a percentage of the maximum hyperpolarization (9.8±1.1mV) in control. B, Schild regression to PZP from data shown in A. Slope=1.0 (95% confidence limits 0.8-1.2). pK_B=6.64 (6.49-6.79). Dose ratios were determined as described in Methods. Data points are the means at each PZP concentration. C, Regression of pK_B estimates calculated from individual PZP concentration and the resulting dose ratio on the corresponding PZP concentration. No significant regression is obtained (t=0.4, d.f.=9).

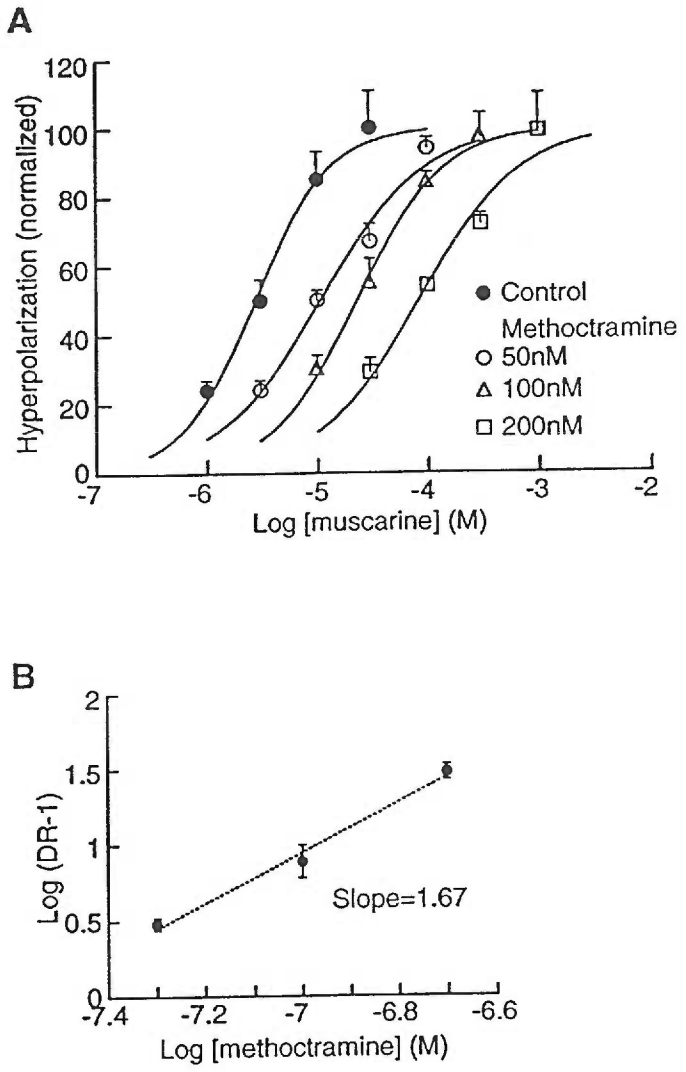

Fig.6 Competitive antagonism of the muscarine-induced hyperpolarization by increasing concentrations of methoctramine. A, Dose-response curves for muscarine in control (●, EC_{50}=2.7 μM, n=8)) and in the presence of 50nM (○, EC_{50}=10.7 μM, n=5), 100nM (△, EC_{50}=23.1 μM, n=4) and 200nM methoctramine (□, EC_{50}=83.9 μM, n=3). Data points represent the percentage of the maximum hyperpolarization in control. B, Schild regression to methoctramine from data shown in A. Filled circles are means at each methoctramine concentration. The dashed line is the best-fit line through the data points (r=0.995). Slope=1.67. X-intercept=-7.56.

CONCLUSIONS AND DISCUSSION

Two types of cells: Primary cells and secondary cells

In the present studies, two distinct cell types, primary and secondary cells, have been distinguished in the NRM according to the intrinsic membrane properties, the neurochemical properties of their synaptic inputs and neurotransmitter-specific immunoreactivity.

Primary cells comprised approximately 80% of total cell population studied. These cells had input resistance of around 180 MΩ, did not fire action potentials spontaneously and had action potential duration of 1-2.4 ms. The fast synaptic potentials were mediated by both GABA and excitatory amino acids (EAA). The GABA-mediated synaptic potential constituted 73% of the total synaptic potential in amplitude and was completely blocked by bicuculline, indicating that $GABA_A$ receptors were involved. Primary cells were generally oval or multipolar and were larger in size (29x18 μM). The majority of these cells (>90%) were immunoreactive for 5-HT and therefore were presumably serotonergic cells.

Secondary cells had higher input resistance (350 MΩ) than primary cells, usually rested at less negative membrane potentials and often were spontaneously active. The action potential duration in these cells was relatively narrow (<1ms). The fast excitatory postsynaptic potential (EPSP) was predominantly mediated by EAA. Secondary cells were round and smaller in size (24x13 μM). Most of these cells (90%) did not stain for 5-HT, thus appearing to be non-serotonergic cells.

As discussed in the foregoing manuscript, the percentage of the 5-HT-containing cells found here is higher than that reported in previous anatomical studies. One possible

explanation is that the location of the recording site was in the part of the nucleus where it contains a large population of 5-HT-containing neurons. Alternatively, it could result from the tendency of microelectrodes to impale larger sized primary cells more successfully. The transmitter that secondary cells contain is unknown at present. GABA is the most likely candidate transmitter at least in some of these cells (see below). Subtypes in each type of cells probably exist and this would require further characterization of the differences in the neurotransmitter content and in functional properties.

Opioids depress the GABA-mediated synaptic potentials in primary cells

It has been demonstrated in present studies that opioids have no effect on the resting membrane potential but depress the amplitude of the GABA-mediated inhibitory postsynaptic potential (IPSP). This presynaptic effect of opioids was mediated through a μ-opioid receptor. There is ample anatomical evidence supporting this action of opioids: In addition to the μ-opioid binding sites, abundant GABA-containing terminals have been found in the NRM (Mugnaini and Oertel, 1985). A recent immunocytochemical study has demonstrated that approximately 50% of synaptic inputs onto the raphe-spinal projection neurons in the NRM are GABA-immunoreactive (Cho and Basbaum, 1991).

Since opioids do not affect the EPSP and the resting membrane potential, opioid inhibition of the GABA IPSP in primary cells would be expected to increase the activity of these cells by disinhibition. This action of opioids could serve as the physiological evidence for opioid-mediated excitatory effects in the CNS and could be the mechanism underlying opioid activation of the descending inhibition systems in the NRM. Consistent with this hypothesis is the behavioral observation that microinjection of the $GABA_A$ receptor antagonist bicuculline into the NRM had an antinociceptive effect (Drower and Hammond, 1988). Functionally, the 5-HT-containing primary cell corresponds to the Off-cell which shows a pause in firing during the tail-flick, as characterized by Fields et al (1983). Thus,

both the primary cell and the Off-cell would be activated by opioids and are thought to be responsible for the descending inhibition. Interestingly, in lightly anesthetized rats, iontophoretic application of morphine has been found to have no effect on spontaneous activity in the Off-cell (Heinricher et al., 1992), but iontophoretic bicuculline eliminates the pause in Off-cell firing just prior to the tail-flick (Heinricher et al., 1991). These results further support the hypothesis that these NRM cells that are activated by opioids are under an inhibitory control by GABAergic inputs. The finding that most primary cells are 5-HT-immunoreactive provide physiological evidence indicating that the 5-HT-containing neurons in the NRM could be activated by opioids and therefore modulate the spinal nociceptive transmission in opioid analgesia.

Opioids hyperpolarize secondary cells by increasing a potassium conductance

Present results have shown that opioids acting on μ-opioid receptors hyperpolarize most of the secondary cells and eliminate their spontaneous activity. This opioid-induced hyperpolarization was mediated through an increase in a potassium conductance. The majority of the secondary cells did not contain 5-HT. Although the neurotransmitter these neurons contain has not been identified yet, there are several lines of evidence suggesting that at least some of them may be GABA-containing neurons: Abundant GABA-containing cell bodies and terminals are present in the NRM (Nagai et al., 1985; Jones et al., 1991); Numerous GABAergic neurons in the NRM project to the spinal cord (Jones et al., 1991), but only few GABA-immunoreactive neurons in the midbrain PAG, a major source of the afferents into the NRM, have been found to project to the NRM (Reichling and Basbaum, 1990); Opioids hyperpolarize the GABA interneurons in the rat hippocampus (Madison and Nicoll, 1988). Thus, it is hypothesized that at least some of the secondary cells are intrinsic inhibitory GABA-containing cells and constitute the source of the GABAergic synaptic inputs onto the primary cells. Opioids could disinhibit primary cells by

presynaptic inhibition of these secondary cells.

Secondary cells may be On-cells. The latter were inhibited by both systemic (Fields et al., 1988) and iontophoretic (Heinricher et al., 1992) application of opioids in lightly anesthetized rats. Using confocal microscopy, Mason et al (mason et al., 1992) have shown that there is significantly greater density of enkephalin-immunoreactive appositions onto the On-cell than onto other types of cells in the NRM. Thus, secondary cells could presumably function as inhibitory interneurons that contribute to the control of the primary cell activity. Opioids produce analgesia by activating the descending inhibitory pathways from primary cells through disinhibition.

5-HT hyperpolarizes most cells in both cell types by increasing an inwardly rectifying potassium conductance

Present studies have elucidated that in both primary and secondary cell groups, 5-HT, acting on 5-HT_{1A} receptors, hyperpolarizes majority (>80%) of the cells in the NRM. The 5-HT-induced hyperpolarization was mediated through an increase in an inwardly rectifying potassium conductance. Additionally, it appeared that in secondary cells, opioids- and 5-HT-induced hyperpolarizations resulted from an increase in the same potassium conductance. This action of 5-HT through 5-HT_{1A} receptors is similar to that on dorsal raphe neurons and has been well characterized previously (Williams et al., 1988; Pan et al., 1989).

In primary cells, a 5-HT-mediated IPSP was evoked following local electrical stimulation and was blocked by the 5-HT_{1A} receptor antagonist. These results suggest that there is an inhibitory serotonergic input onto these 5-HT-containing primary cells. Therefore it seems that the 5-HT_{1A} receptor on 5-HT-containing primary cells may function as an autoreceptor to regulate activity, as has been proposed previously in dorsal raphe nucleus (Wang and

Aghajanian, 1978). The function of the 5-HT_{1A} receptor on secondary cells is unclear. Although there is an extrinsic source of 5-HT-containing terminals in the NRM (Beitz, 1982), one possibility is that these secondary cells may be under an inhibitory serotonergic modulation synaptically from 5-HT-containing primary cells intrinsic to the NRM. If this is the case, the inhibition mediated by the 5-HT_{1A} receptor on secondary cells and the inhibition by $GABA_A$ receptor on primary cells would reciprocally coordinate the activity of these two cell types. Activation of one type would inhibit the other, as these two types of cells have opposite actions on nociceptive transmission. This reciprocal activity has been found between Off- and On-cells in lightly anesthetized rats (Barbaro et al., 1989).

Thus, the presence of 5-HT_{1A} receptors on both primary and secondary cells may explain, at least in part, the previous conflicting reports on the effect of exogenous 5-HT on nociceptive threshold. As discussed previously, 5-HT-containing primary cells could be activated by analgesic opioids. This is further supported by a behavioral observation that systemic application of 5-HT_{1A} receptor agonists that would inhibit primary cells (and secondary cells as well) attenuated opioid-induced analgesia (Millan and Colpaert, 1991).

Muscarine hyperpolarizes a subpopulation of the NRM cells by activating an M_2 muscarinic receptor

Present studies have provided pharmacological and physiological evidence suggesting that an M_2 muscarinic receptor is coupled to an inwardly rectifying potassium conductance in the NRM cells. This coupling has not been clearly characterized in the CNS before. Due to recent new pharmacological definition of muscarinic receptor subtypes, the previous defined M_2 subtype that mediates various responses in the CNS has to be reconsidered in classification. Moreover, the potassium conductance that has been shown to be coupled to the previous defined M_2 subtype is linearly dependent on the membrane potential (Egan and North, 1986; McCormick and Prince, 1985). In a recent report on rat pontine reticular

formation neurons, the potassium conductance that was activated by muscarinic agonists rectified inwardly, but the receptor subtype was not identified (Gerber et al., 1991). It has been found in present studies that in a subpopulation of primary cells, muscarine hyperpolarizes all the cells. These results imply that the activity of a subpopulation of 5-HT-containing primary cells is under a cholinergic inhibitory postsynaptic modulation. This is supported by a recent behavioral study where microinjection of the M_2 receptor antagonist methoctramine into the NRM to block the cholinergic inhibition on the NRM neurons produces an antinociception. This finding implicates that this cholinergic inhibitory modulation may be tonically active. Whether these muscarine-responding primary cells contain ACh or not and the functional difference between these cells and other non-responding primary cells are unknown. Further studies are necessary to elucidate the functional significance of muscarine-mediated inhibition and excitation in secondary cells.

Summary

In summary, present studies have characterized two types of cells in the rat NRM, primary cells and secondary cells. Opioid inhibition of the GABA IPSP in primary cells provides physiological evidence for the excitatory effect of opioids through disinhibition in the CNS and is suggested to be the mechanism underlying opioid activation of the descending inhibition systems in the NRM in opioid analgesia. Secondary cells that are hyperpolarized by opioids could be the candidate for the intrinsic GABA neurons that synaptically innervate primary cells. 5-HT-containing primary cells that are activated by analgesic opioids through disinhibition may regulate the activity by autoinhibition through 5-HT_{1A} receptors. In a subpopulation of primary cells, an M_2 muscarinic receptor and a 5-HT_{1A} receptor are coupled to the same inwardly rectifying potassium conductance, indicating an inhibitory cholinergic as well as a serotonergic modulation on these cells.

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APPENDICES

Appendix 1: Effects of noradrenaline on the neurons in the NRM *in vitro*

As discussed in the introduction, previous physiological and behavioral studies have shown that the cellular activity in the NRM are affected by exogenously applied noradrenaline (NA). Manipulation of the noradrenergic transmission in the NRM has an action on spinal nociceptive transmission. In this study, direct actions of NA on primary and secondary cells in the NRM were examined in a slice preparation. The ionic mechanisms underlying the NA actions were investigated.

Preliminary results

Different responses elicited by noradrenaline in two types of cells

Effects of NA were tested on the resting membrane potential in both primary cells and secondary cells. In primary cells, NA (10μM) had a complex action which remained in the presence of tetrodotoxin (TTX, 1μM)) to eliminate indirect presynaptic actions (Fig.1A). NA caused a depolarization that declined in amplitude during NA application. In the course of washing out NA, the cell displayed a rebound depolarization which varied in amplitude among cells and then recovered to control membrane potential. In contrast, in secondary cells, NA (10 μM) produced a depolarization that often resulted in firing of repetitive action potentials and the cell recovered upon washing out NA (Fig.1B). This NA-mediated depolarization was mimicked by the selective α_1-adrenoceptor agonist phenylephrine (PE, 300nM - 3μM) and blocked by the selective α_1 receptor antagonist prazosin (1 μM) (data not shown), suggesting that the depolarization was mediated by α_1-adrenoceptors.

NA activation of both α_1 and α_2 adrenoceptors in primary cells.

The action of NA in primary cells was studied by using selective adrenoceptor agonists. In a primary cell shown in Fig.2, PE (0.6 μM) caused a depolarization that recovered upon washing PE without rebound depolarization, indicating an α_1-adrenoceptor-mediated response. In the same cell, UK14304, a selective α_2 adrenoceptor agonist, produced a hyperpolarization that was not affected by the 5-HT_{1A}/5-HT_2 receptor antagonist spiperone (1 μM), but blocked by the selective α_2 receptor antagonist idazoxan (1 μM), indicating the involvement of an α_2 adrenoceptor. These results suggest the co-existence on these primary cells of both α_1 adrenoceptors mediating a depolarization and α_2 adrenoceptors mediating a hyperpolarization. This was further demonstrated by the experiments where actions of NA were studied in the presence of the α_1 or α_2 receptor antagonists. In the presence of idazoxan (1 μM) to block the α_2-mediate response (Fig.3Ab), NA (10 μM) produced a depolarization without the rebound depolarization as seen in the absence of the antagonist (Fig.3Aa). In another primary cell, NA elicited a typical response in control (Fig.3Ba), but hyperpolarized the cell after blocking the α_1 receptor by prazosin (1 μM, Fig.3Bb). These results indicate that NA elicits the complex response by activating both the α_1 and α_2 adrenoceptors that are co-localized on these primary cells.

The α_1-depolarization is due primarily to a decrease in a potassium conductance

In single electrode voltage clamp, NA induced an inward current at the resting membrane potential with a reduced membrane conductance in both primary and secondary cells (Fig.4A). The inward current declined in amplitude at more negative potentials than the resting potential, but no reversal in polarity was ever observed. Similar current was induced by PE (3 μM) (Fig.4B). These results suggest that more than one conductance

were activated by α_1 adrenoceptor activation. In fact, this action of α_1 agonists has been observed in dorsal raphe neurons. As it is practically much more difficult to get recordings from the NRM cells, further studies of the mechanism underlying the α_1-mediated depolarization were carried out in dorsal raphe neurons using whole-cell recording technique (Pan et al., 1993). Figure 5 shows some of the results in that study. With whole-cell recordings in current-clamp mode, PE (10 μM) caused a depolarization similar in amplitude to that with intracellular recordings. In voltage-clamp, PE (10 μM) produced an inward current and decreased the resting membrane conductance. The PE-induced current reversed polarity at the potassium equilibrium potential (Fig.5A). The reversal potential was dependent on the extracellular potassium concentration and was shifted to the less negative potentials when extracellular potassium concentration was increased from normal (2.5mM) to 6.5 mM or 10.5 mM (Fig.5B). These results suggest that the depolarization mediated by α_1 adrenoceptors involves primarily a decrease in the resting potassium conductance.

The α_2-hyperpolarization results from an increase in a potassium conductance.

In voltage clamp, UK14304 (300nM) caused an outward current associated with an increase in membrane conductance in a primary cell (Fig.4C). The current reversed polarity at near potassium equilibrium potential (-108mV), indicating the involvement of a potassium conductance.

Conclusions and discussion

Interactions between the α_1 and the α_2 responses in primary cells

Preliminary results have shown that in primary cells, both an α_1-receptor-mediated

depolarization and an α_2-mediated hyperpolarization can be elicited. It appears that the complex response induced by NA is probably a mixture of α_1- and α_2-mediated responses: NA caused the initial α_1-mediated depolarization that was usually dominant and had faster onset at 10 μM (Fig.1A, 3Aa). Then the depolarization declined as a result of the counteraction by the development of an α_2-mediated hyperpolarization which had slower onset rate (Fig.3Bb). Upon washing NA, it took much longer for the depolarization to recover in the presence of the α_2 response (Fig.3A), possibly due to interactions at the intracellular second message level. The hypothesis that the NA response is due to interactions between α_1 and α_2 actions is supported by the observation that after blockade of one receptor, NA induced the response mediated by the other (Fig.3).

It was observed that there was a considerable variability in the amplitude of the NA-induced depolarization and the rebound depolarization among primary cells. The reason for this difference is unknown at present and further studies are needed to establish the relative potencies of NA on α_1 and α_2 receptors in these primary cells. Functionally, it is possible that the sensitivity of both α1 and α2 adrenoceptors in these cells may be under regulation at the single cell level to modulate its functional state in the nociceptive pathways. In contrast, secondary cells were uniformly depolarized by NA, implicating a consistent, stronger excitatory effect on the activity of these cells. This is consistent with a previous in vivo observation that nociceptive reflexes were enhanced in awake rats following microinjection of an α_1-adrenergic agonist into the NRM (Fields et al., 1991).

Ionic mechanisms of the α adrenoceptor-mediated responses

The coupling of the α_2 receptor activation to an increase in a potassium conductance has

been described in several regions in the CNS (Surprenant, 1989; Summers and McMartin, 1993). The ionic mechanism involved in the α_1-adrenoceptor-mediated depolarization has not been clarified in the CNS. As seen in other studies (Legendre et al., 1988; McCormick and Prince, 1988), no reversal in the polarity of the α_1 agonist-induced current was observed in this study. The present results with whole-cell recordings have demonstrated both the reversal potential near the potassium equilibrium potential and its dependence on the extracellular potassium concentrations. These results suggest that a decrease in the resting potassium conductance is the primary mechanism for the depolarization. it is presently not known why the current reversal can be observed with whole-cell recordings but not with microelectrode recordings. It is possible that the whole-cell recording may cause the dialysis of the intracellular transduction system which prevents the reversal of the current polarity using microelectrodes. Activation of α_1 adrenoceptors has been shown to result in an increase in intracellular free calcium level through the phospholipase C-IP_3 pathway (Summers and McMartin, 1993). However, it appears that the α_1-mediated depolarization is independent of this pathway and of the intracellular calcium increase, as a similar a1-mediated depolarization was observed with a recording pipette containing the calcium chelator BAPTA (20 mM) (Pan et al., 1993). These issues have been further investigated in that study on dorsal raphe neurons.

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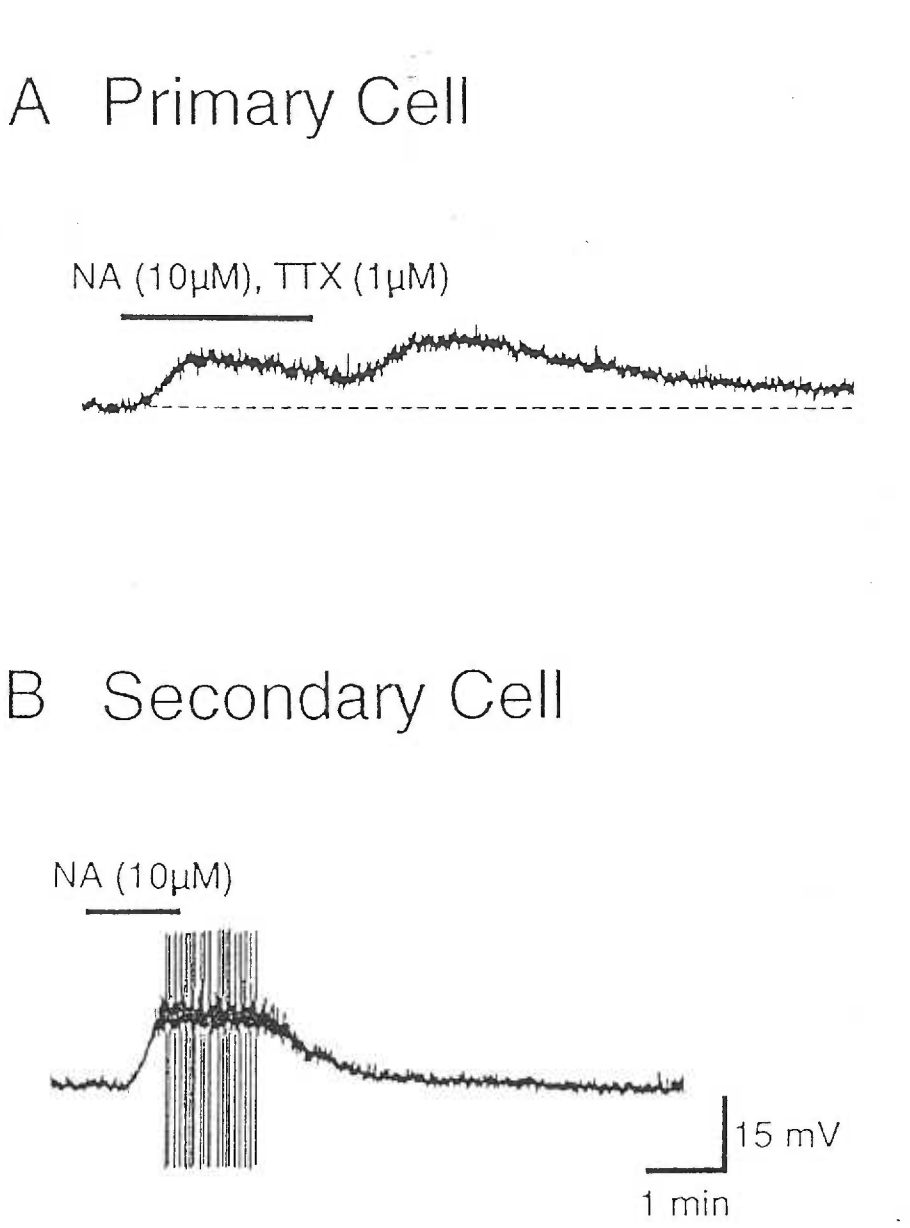

Fig.1 Different responses elicited by noradrenaline (NA) in primary and secondary cells. **A**. In the presence of tetrodotoxin (TTX) to eliminate any indirect presynaptic effect, NA induced a depolarization followed by a rebound depolarization upon washing NA in a primary cell. **B**. In a secondary cell, NA produced a depolarization which resulted in repetitive firing and washed out directly.

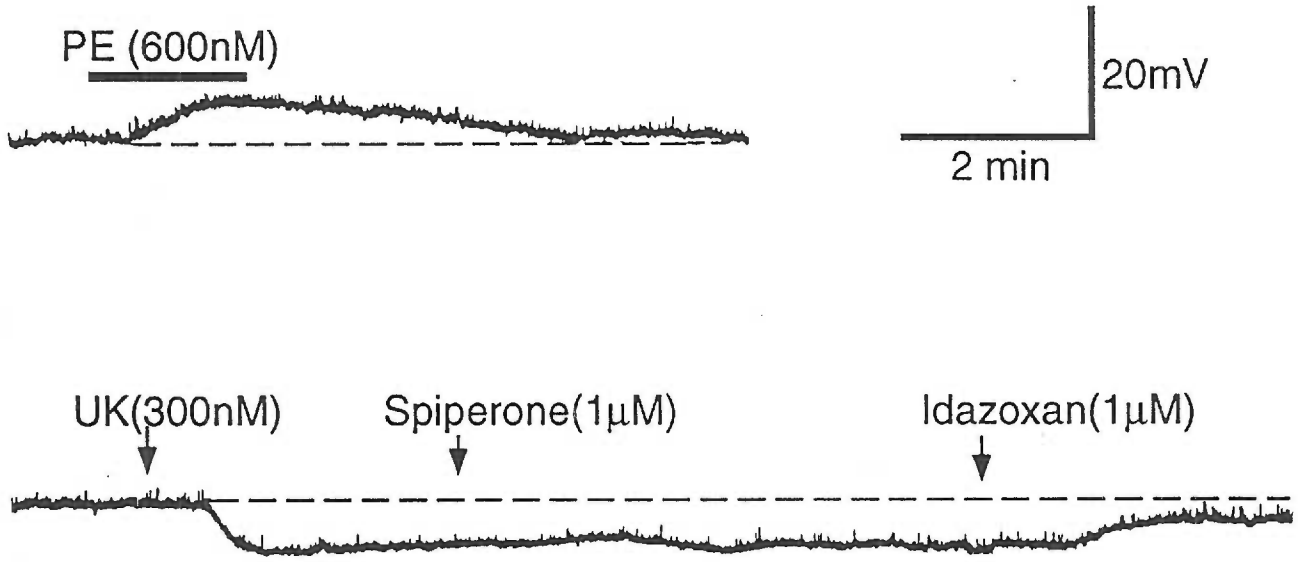

Fig.2 Existence of both an α_1- and an α_2-adrenoceptor-mediated responses in a primary cell. The cell was depolarized by phenylephrine (PE), a selective α_1 receptor agonist. In the same cell, UK14304, a selective α_2 receptor agonist, caused a hyperpolarization that was blocked by idazoxan, a selective α_2 receptor antagonist, but not by spiperone, a 5-HT_{1A}/5-HT_2 receptor antagonist.

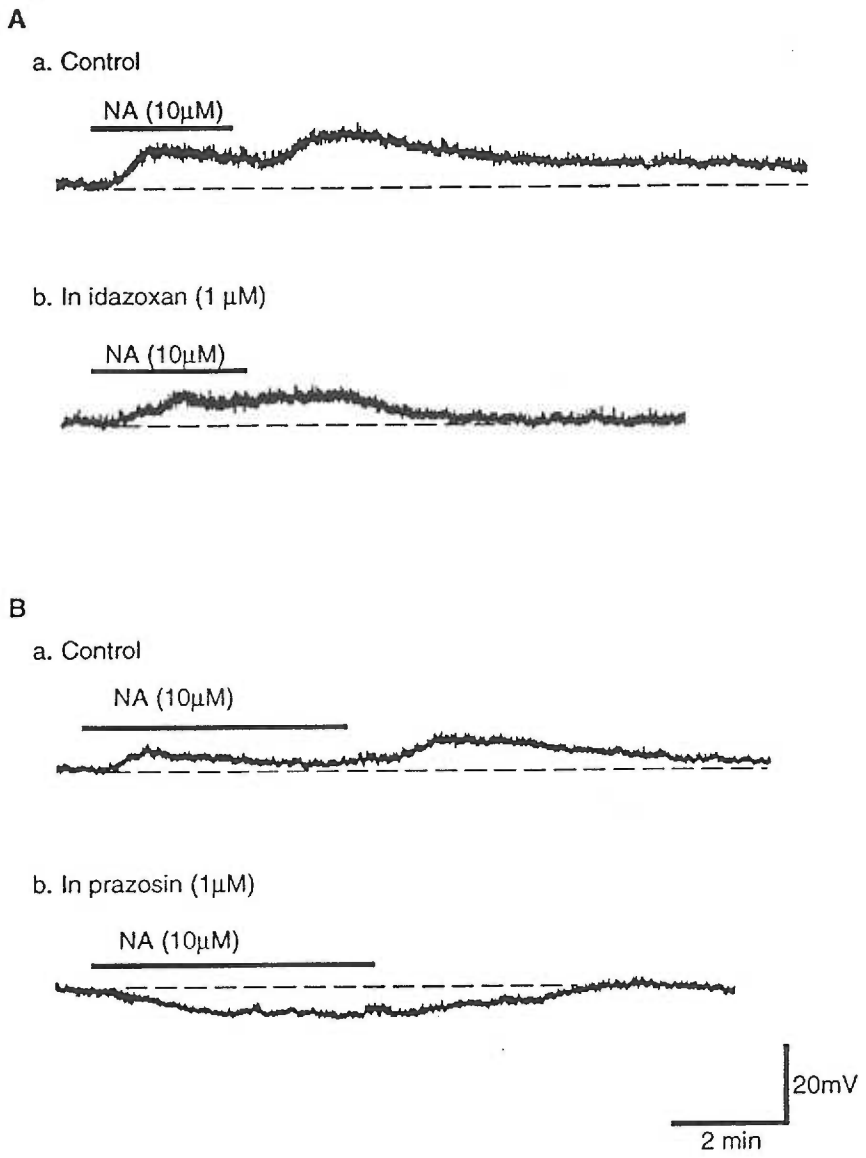

Fig.3 Interactions between the α_1- and the α_2-adrenoceptor-mediated responses in primary cells. A. NA elicited a complex response in a primary cell in control (a), but induced a normal depolarization after blockade of the α_2 receptors with idazoxan in the same cell (b). B. In another primary cell, NA had the complex depolarizing effect in control (a). In the presence of prazosin to block the α_1 receptors, NA produced a hyperpolarization. Note the difference in amplitude of the depolarizations by NA (10 μM) in these two primary cells shown in A.a and B.a.

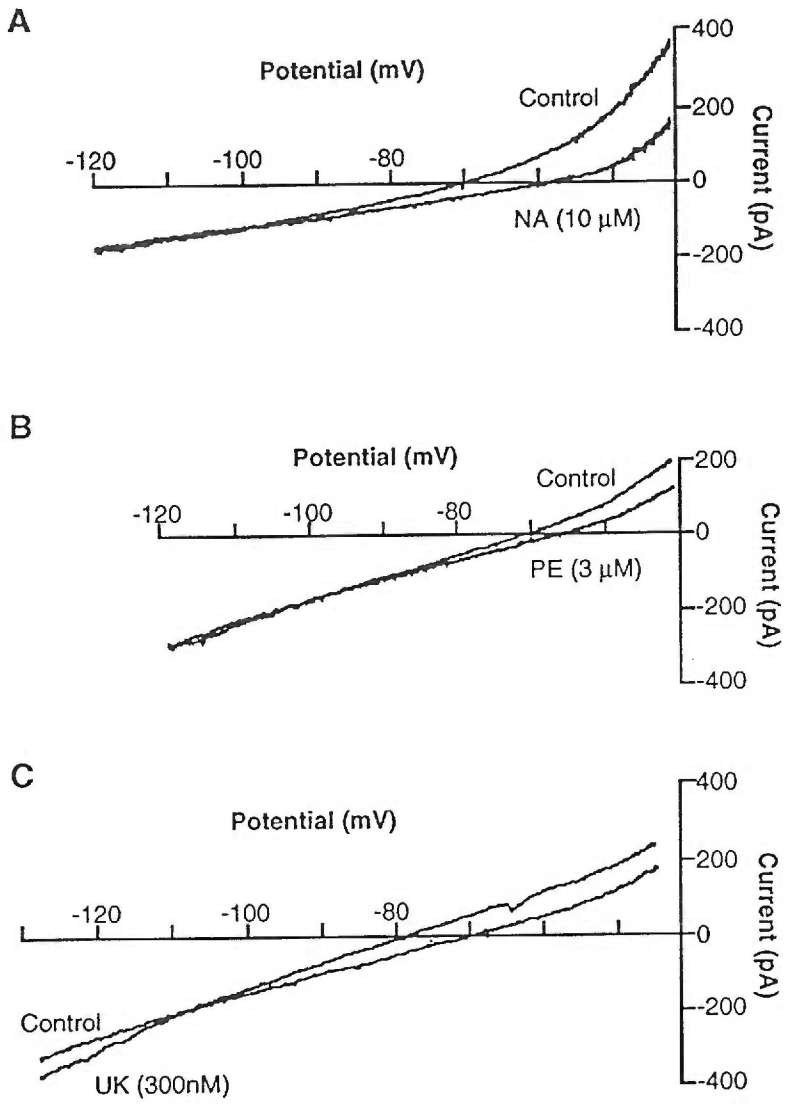

Fig.4 Current-voltage plots with single electrode recordings in the absence and presence of adrenoceptor agonists in primary cells. A. NA induced an inward current at the resting membrane potential and the current declined in amplitude but did not reverse polarity at more negative potentials. B. In another primary cell, selective activation of α_1 receptors by PE caused a similar inward current to that induced by NA. C. UK14304 produced an outward current at the resting potential in a primary cell. The current reversed polarity at around potassium equilibrium potential (-108 mV).

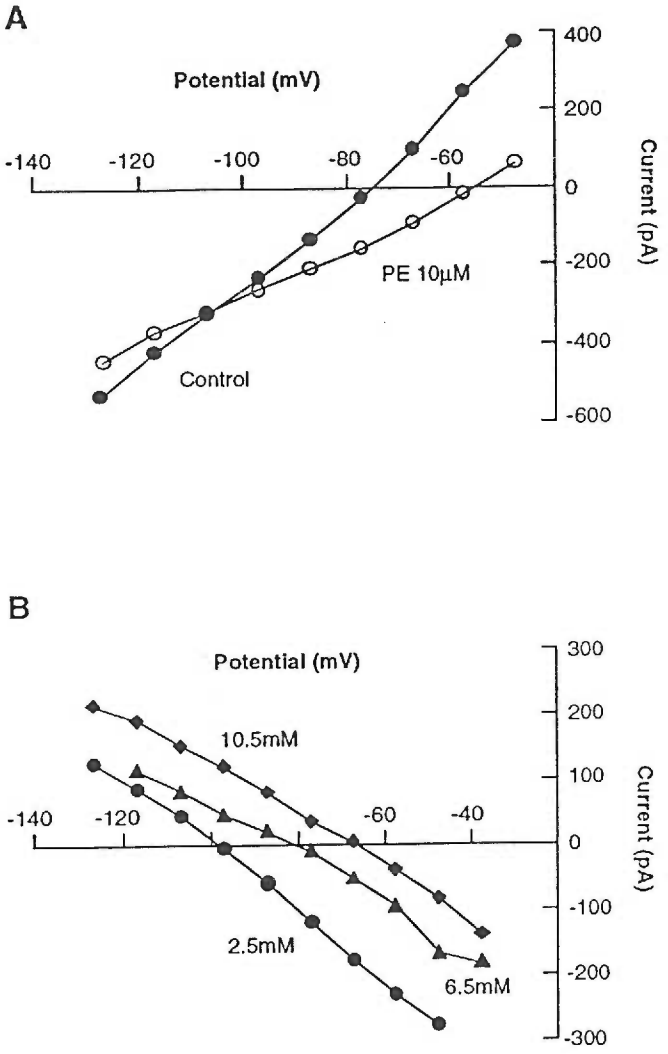

Fig.5 Current-voltage plots with whole-cell recordings from dorsal raphe neurons. A. PE caused an inward current at the resting membrane potential and the current reversed polarity at near the potassium equilibrium potential (-106 mV) in a dorsal raphe neuron. B. Subtracted PE currents from another cell. The PE current reversed polarity at -99 mV in normal extracellular potassium concentration (2.5 mM) and the reversal potential was shifted to -81 mV and -66 mV when the extracellular potassium concentration was increased to 6.5 mM and 10.5 mM, respectively.

Appendix 2: Other accomplishments

1) **Pan ZZ**, Tong G, Jahr CE (1993) A false transmitter at excitatory synapses. **Neuron** (accepted).

2) **Pan ZZ**, Grudt T, Williams JT (1993) Alpha 1-adrenoceptors in rat dorsal raphe neurons: Depolarization and regulation of activity. (in preparation).