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Tachykinin receptor role in the synaptic transmission from dorsal root fibres to motoneurones: an intracellular study

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ABSTRACT

In the spinal cord of neonatal rat the involvement of tachykinins in synaptic transmission from dorsal root fibres to motoneurones was studied by using intracellular microelectrode recording. First, it was shown, by application of specific tachykinin agonists, that all types of tachykinin receptor are functional in the spinal cord of neonatal rat. Nevertheless, only NK_1 receptors are consistently present on motoneurones as revealed by the persistence of the responses in the presence of TTX (1μM). These responses consisted of membrane depolarisation with an apparent rise in input resistance which was voltage-sensitive. In addition a minority of motoneurones (40%) possessed NK_3 receptors. Several antagonists to NK_1 receptors were tested. RP 67580 and CP 99994 appeared to be ineffective while SR 140333 blocked completely the responses to the NK_1 receptor agonist SPMeO. In addition, the NK_2 receptor antagonist SR 48968 reduced (by 50%) responses to the NK_2 receptor agonist [Ala]NKA while responses to SPMeO were almost unchanged. Nevertheless, no effects of SR 48968 on synaptic responses was detected indicating a minimal role of SR 48968 -sensitive NK_2 receptors in synaptic transmission from dorsal roots to motoneurones. Conversely, SR 140333 reduced spontaneous activity and fast synaptic potentials. It is suggested that there was a tonic activation of NK_1 receptors in the spinal cord of neonatal rat. Furthermore, SR 140333 depresssed slow postsynaptic potentials in the majority (10/14) of cells and decreased to 67% (n = 14) the rate of rise of cumulative depolarisation induced by 1 Hz pulse trains. The block by SR 140333 of the rate of rise was voltage independent while the rate of rise itself was enhanced by membrane depolarisation. These results suggest that NK1 receptors on motoneurones could be partially responsible for such an unusual voltage dependence of the rate of rise, the magnitude of which can predict the presence of action potential windup, an important manifestation of sensitisation of nociceptive pathways.

The rate of rise of cumulative depolarisation was not reduced by NMDA receptor antagonists (actually it was increased by 20 %) and it retained similar voltage dependence. This observation is incompatible with hypothesis that the unusual voltage dependence of rate of rise of cumulative depolarisation can be explained by voltage dependent relief of magnesium block relief from NMDA receptor channels. The action of SR 140333 on slow synaptic potentials and rate of rise persisted in the presence of NMDA or non-NMDA receptor antagonists. In addition, there was positive correlation ($r = 0.75$) between the depolarising tails (600 - 800 ms after stimulus) of slow synaptic potentials in the presence of NMDA receptor antagonists and the rate of rise of cumulative depolarisation. Moreover, these tails had voltage dependence similar to the one of the rate of rise of cumulative depolarisation and these tails were reduced by SR 140333. Taken together these data suggest that NK1 receptors, independent from glutamate receptors, were involved in synaptic transmission from dorsal roots to motoneurones and contributed to summation of slow potentials partially unmasked in the presence of NMDA receptor antagonists. The experiments with different stimulus strengths in the presence of NMDA receptor antagonists revealed a clear threshold for generation of a high rate of rise of cumulative depolarisation (leading to the action potential windup) regardless of the area of single polysynaptic potentials. These findings indicate that activation of particular fiber class (and related transmitter release) rather than mere depolarisation is important for producing a high rate of rise of cumulative depolarisation. Since the action potential windup is considered as an experimental model for the central component of pain induced sensitisation, and the windup is correlated with the presence of high rate of rise of cumulative depolarisation, the findings of these thesis suggest that slow neurotransmitters (one of them is substance P) play a major role in the induction of central component of sensitisation as they mediate slow synaptic potentials capable to summate at low (0.5-2Hz) frequencies.

Introduction

The main purpose of the nervous system is to convey and process information. Even though electrical synapses are present in the central nervous system the main mode of communication between neurones is by chemical substances- mainly via neurotransmitters. There are many messages to be communicated between neurones and consequently many chemical compounds are used as neurotransmitters (Hartzell, 1981). It is not surprising that peptides are also used for this purpose as it is possible to have a huge number (~24^{10} or in other terms ~10^{15}) of them on the basis of a single mechanism of protein/olygopeptide synthesis. This feature makes them the most numerous group of neurotransmitters (Rang and Dale, 1991). Nevertheless, only in the 70s the structure of the first peptides found in the brain (enkephalins and substance P) was determined and the concept of peptide as a possible neurotransmitter was introduced (Snyder, 1980). Many new structures of brain derived peptides have been described and many of them exhibit a powerful physiological action like smooth muscle contraction, vasodilation etc. It was shown that peptide binding receptors are present in the central nervous system and that peptides could be released upon stimulation (Krieger, 1983). In addition, exogenous application of peptides could excite/inhibit central neurones directly and indirectly. Hence, it was suggested that they can act as neurotransmitters in the central nervous system (Snyder, 1980). Nevertheless, there are few direct observations of peptide-mediated synaptic potentials in neurones which could confirm their neurotransmitter role in the central nervous system. There are several reasons for that. First of all, their responses are slow. This makes the responses skewed and small, and therefore difficult to relate to peptide release. The slowness of these responses is for two

reasons. First, since peptides bind to G protein coupled receptors (there is no report of peptide receptors directly coupled to channel pores), the time course of the response therefore depends on intracellular biochemical reactions and not only on the binding to receptors; consequently the time needed for the response increases from milliseconds to hundreds of milliseconds and even more. In addition, in most cases the peptides are quite long (9 or more amino acid residues) and the bulkiness of the molecule slows down diffusion. On the other hand, the role of the peptides in synaptic transmission could be completely different from that of classical transmitters and their role could be much more variable. Hence, the study of neuropeptide action could help to complete the picture of the brain function. A classical example of endogenously occurring peptide is substance P which belongs to the tachykinin peptide family and is one of the best studied peptides (Otsuka and Yoshioka., 1993)

SUBSTANCE P AND TACHYKININS

Substance P has long been known to be a bioactive agent derived from the brain and able to lower blood pressure and stimulate various organs (intestine, blood vessels, etc.). Only in 1971 the structure of SP was reported (Chang et al, 1971) and after ten years it was included, together with later discovered peptides, into a family under the broad term of tachykinins (Maggio, 1988). This name is derived from Greek and means 'quick action' as tachykinins, in comparison to other functionally related peptides such as bradykinin ('slow action'), act faster. Nevertheless, the fastest onset of SP action is measured in seconds, much slower than that for fast neurotransmitters, for example glutamate (Urban and Randic, 1984). Tachykinins exert a wide variety of actions: they induce smooth muscle contraction, endothelium dependent vasodilation, plasma protein extravasation, mast cell degranulation,

direct stimulation of secretion and other neurotransmitter (dopamine) production, recruitment of inflammatory cells (Maggi et al, 1993). In addition, many neurones, mainly in the periphery, can change their membrane potential (in the majority of the cases by depolarisation) due to direct action of tachykinins (via intracellular messengers). All these responses can be exemplified by substance P (Otsuka and Yoshioka, 1993). As such different actions cannot be the basis for tachykinin classification, an approach based on their chemical structure has been used for this purpose.

All peptides of the tachykinin family share a common N end amino acid sequence: Phe-Phe/Val/Ile/Tyr-Gly-Leu-Met-NH2 and it is the basis for their assignment to this family. At the present time five mammalian (substance P, neurokinin A, neurokinin B, neuropeptide K, neuropeptide γ) and several nonmammalian tachykinins (kassinin, eledoisin, physalaemin) are known (Regoli et al, 1994). There are reports of other tachykinin-like peptides and new discoveries are possibly awaiting us.

Mammalian tachykinins derive from two preprotachykinin (PPT) genes which evolved from a common ancestor gene by duplication (Mussap et al, 1993): the PPT-A from which substance P, NKA and the extended NKA homologues NPK and NPγ are generated, and the PPT-B which encodes NKB. The PPT-A precursor is translated into four PPT-A mRNAs as a consequence of alternative RNA splicing events: α-PPT-A mRNA (abundant in the central nervous system), which generates substance P; β-PPT-A mRNA, which contains substances P, NKA and NPK; γ-PPT-A mRNA, which generates substance P, NKA and NPγ; and δ-PPT-A mRNA which encodes substances P and a peptide of 22 amino acid residues (Harmar et al, 1990). PPT-A and PPT-B mRNA contains precursors of tachykinins and the functional peptides are liberated by the action of specific processing proteases followed by COOH-terminal amidation after cleavage of the Lys-Arg ending (Regoli et al, 1994).

TACHYKININ RECEPTORS

Most of the actions of tachykinins are due to binding to tachykinin receptors. Already in 1973 Erspamer and co-workers were among the first to observe markedly different potencies for substance P and nonmammalian tachykinins with various bioassays; subsequent work confirmed this (Mussap et al, 1993). Later, a consensus was reached to distinguish three main types of tachykinin receptor according with the order of potency of neurokinins: substance P preferring receptors are named NK1, NKA-preferring ones are named NK2, and NKB preferring ones are NK3. Successful cloning of all three receptor subtypes confirmed this classification. Nevertheless, there are indications that this classification is oversimplified (Maggi et al, 1993) and, in addition, some non-tachykinin receptor related action of substance P (for instance, the modulation of nicotinic acetylcholine receptor) has been reported (Maggi et al, 1993). The issue of receptor subtypes is related to the problem of specificity of agonists and antagonists and will be discussed in detail later in the present thesis.

The activation of tachykinin receptors stimulates phospholipase C leading to phosphoinositide breakdown and elevation of intracellular calcium (Otsuka and Yoshioka, 1993). In some cases adenylate cyclase stimulation and cAMP formation have been observed and the intracellular parts of the receptor responsible for the effect have been determined (Brodbeck et al,1994). Nevertheless, it can be concluded that (according to our current knowledge) tachykinin receptor subtypes activate similar second messenger systems. Therefore, the difference in their responses at the cellular level should depend more on later steps in the intracellular signalling cascade.

At cellular level the effects of tachykinin receptor activation range from membrane potential changes to secretion and contraction. In the case of neurones we are interested

mainly in three phenomena (all of them can change the properties of the neuronal network): firstly, membrane potential changes; secondly, direct stimulation or facilitation of transmitter release; and thirdly, modulation of the responses to other transmitters. Only the first type of responses is usually considered as neurotransmission while the later two ones are included into neuromodulation. All these types of action have been reported for tachykinins (Murase et al, 1982, Suzuki et al, 1993; Cumberbatch et al, 1995;). It implies that the action of tachykinins can be very complex. There are several peripheral neuronal systems (mesenteric plexus, dorsal root ganglia) where the mechanisms of tachykinin action have been studied in more detail and showed that slow postsynaptic potentials are possibly mediated by tachykinins (Otsuka and Yoshioka, 1993) and hence the peptide is acting as a classical neurotransmitter. In these cases the slow potentials can be observed separately and their properties can be studied relatively easy without interference from classical fast neurotransmission. Conversely, in the central nervous system tachykinins, like many other peptides, coexist with fast neurotransmitters (glutamate, GABA; De Biasi and Rustioni, 1988; Kachidian et al, 1991) and, as a consequence of simultaneous release of both transmitters, complex postsynaptic potentials are observed. In some cases (e.g. in dorsal horn neurons upon afferent fiber stimulation) the slow synaptic potentials can be separated from the fast ones by special stimulation protocols (Urban and Randic, 1984). Nevertheless, in most cases these potentials overlap and the question of specific antagonists becomes important as pharmacological block of receptors could allow detecting the role of each receptor type (and by inference of each neurotransmitter) in the generation of postsynaptic potentials.

TACHYKININ AGONISTS AND ANTAGONISTS

The first experiments indicating the existence of multiple substance P receptors were performed in 1973 by Erspamer and co-workers (see Mussap et al, 1993). Nevertheless, it took more than 10 years to elaborate specific agonists for various tachykinin receptor subtypes; finally Regoli and co-workers established the existence of three tachykinin receptor types by using specific agonists (Dion et al, 1987; Drapeau, et al, 1987). Some of these agonists are in current use and they are supposed to be specific. Here is the list of the most popular of them:

for NK1 receptor type- substance P methyl ester, [Sar^9,$Met(O_2)^{11}$]SP, [Pro^9]SP;

for NK2 receptor type- [β-Ala^8]NKA-4-10, GR64349,

[Lys^5MeLeu^9,$Nleu^{10}$]NKA-4-10;

for NK3 receptor type- senktide, [$MePhe^7$]NKB, [Pro^7]NKB.

It should be always borne in mind that all naturally occurring peptides are full agonists for all three receptor types even though with different binding affinity. For instance, substance binds preferentially to the NK1 receptor type but it can activate also NK2 and NK3 receptor types.

Nevertheless, there are still some discrepancies in their selectivity and there are proposals for the existence of a further subdivision of tachykinin receptor classes (for instance, a receptor selective for septide, another tachykinin receptor agonist the selectivity of which is not clear, Maggi et al, 1993). These suggestions became more frequent with the appearance of numerous antagonists as their affinity and potency were quite variable from preparation to preparation. One of the first substance P antagonists was spantide which is still in use in spite of its low selectivity and low stability due to its peptide structure. Only in the early 90s more stable (as they were nonpeptide) and much more specific tachykinin receptor antagonists

appeared. Their discovery was due to the introduction of new methods of search based on quick parallel screening of whole classes of substances generated by nonspecific chemical reactions (which produce a whole range of chemically related substances). CP 96 345 (Snider et al, 1991) was the first of that generation of antagonists but it was also the first to show all pitfalls of such agents. It was discovered soon that it has 100 times lower affinity for NK1 receptors in the rat and the mouse in comparison to the human and most other species (Barr and Watson, 1993); secondly, its nonspecific activities (binding to calcium channels and non-specific inhibition of synaptic transmission, Guard and Watling, 1992, Wang et al, 1994) were reported. The availability of a range of antagonists to NK2 receptors even led to the proposal of the existence of two subclasses of these receptors- 2A and 2B (Maggi et al, 1993). Molecular biology studies showed that the binding site for most of these antagonists slightly differed from the binding site for the agonist (Gether et al, 1993), thus explaining different pharmacological profiles for agonists and antagonists. Nevertheless, new methods of antagonist synthesis proved to be effective and by now we have a whole range of stable and highly specific antagonists for the main classes of the tachykinin receptor (at the time of the present study antagonists to NK3 receptor type are still lacking).

In summary, a rapid development in the field of tachykinin agonists and antagonists during the last 15 years made available potent pharmacological tools for detailed studies of the tachykinin role in neurotransmission. The idea that substance P can be a transmitter in the central nervous system (in addition to the periphery) developed gradually and it is worthwhile to present a very brief historical overview of the topic.

DEVELOPMENT OF NEUROTRANSMITTER CONCEPT FOR SUBSTANCE P

Substance P was discovered as an agent with hypotensive and spasmogenic activity that was clearly different from acetylcholine in that it contracted the rabbit jejunum in the presence of atropine (for a historical review see von Euler, 1981). Soon after, Dale suggested that the chemical transmitter secreted from the peripheral ending of a sensory neurone to cause axon-reflex vasodilatation might be the same as the transmitter secreted from the central ending of the same neurone (Dale's principle, Otsuka and Yoshioka, 1993). Since substance P was found to be present in extracts from gut and brain and its action on rabbit jejunum was similar to the vagus stimulation Dale's principle can be considered as the first suggestion that substance P is a neurotransmitter. In 1953 the substance P like activity found in dorsal root tissue was reported to be much higher than in ventral roots which led to the proposal that substance P is a sensory transmitter (Amin et al, 1954; Otsuka and Yoshioka, 1993). It was suggested that substance P could be involved in pain transmission as the dorsal horn is believed to process nociceptive information. Nevertheless, the first attempts to observe responses of central neurones to substance P were unsuccessful (Haefely and Hurlimann, 1962). Only later it was demonstrated by immunohistochemical studies that substance P is found in small diameter fibers only (Hökfelt et al, 1975a and b) and it was observed that this peptide can excite dorsal horn neurones that respond to noxious stimuli (Henry, 1976, Randic and Miletic, 1977, Sastry, 1979). This was strong evidence for the idea that substance P can transmit nociception. Subsequent studies developed the concept of substance P as sensory, mainly nociceptive transmitter. Therefore, the issue of pain and the characteristics of nociceptive transmission will be introduced in more detail.

THE SPINAL CORD AS A PRIMARY SITE OF PAIN PROCESSING

Pain sensation is subjective and therefore difficult to define. One of the definitions can be as signalling of possible tissue damage to the organism. Nociception is the mechanism whereby noxious peripheral stimuli are transmitted to central nervous system. Nociceptors are defined as sensory receptors with high threshold for response to stimulation (indicating that only strong, potentially dangerous stimuli are detected). A general feature which distinguishes the nociceptive system from most sensory systems is the ability to enhance its responsiveness as a consequence of adequate stimulation. This process is called sensitisation and is characterised by lowered response threshold, induction of spontaneous activity, and enhanced responses in the suprathreshold range. There are several types of sensory receptors and they could be subdivided into two major groups : first, low threshold receptors, they convey signals via myelinated large fibers and usually cannot induce pain sensation; second, high threshold receptors which transmit nociception via small (mainly unmyelinated) fibers from the skin to the spinal cord and brainstem. Most of the fibers responsible for nociception end in superficial layers of the dorsal horn (substantia gelatinosa) and in the trigeminal complex of the brainstem. In the spinal cord they make contacts with dendrites of dorsal horn cells, part of which send ascending fibers to the medulla and thalamus where a putative pain detecting nucleus has recently been found (Craig et al, 1994). There are also several descending pathways which control each of these steps in synaptic transmission (part of them exhibit a tonic inhibition of nociceptive neurones). In addition, skin nociceptors are strongly modulated by inflammation. Perhaps the most striking feature of the nociceptive system is its remarkable plasticity which ranges from simple sensitisation to the global rearrangement of sensory fields in the spinal cord (Cook et al, 1987; McMahon et al, 1993; Zieglgansberger and Tolle, 1993; Treede and Magerl 1995;).

The spinal cord is the first compartment of the central nervous system where the nociceptive information is processed and in many cases it is also the immediate site of the response to noxious stimuli as it contains motoneurones which operate the withdrawal reflex. It is believed that some pain related responses are confined to the spinal cord circuitry, as for example, facilitation of withdrawal reflex (Baldiserra et al, 1971). Much processing of this information should occur in the dorsal horn, especially in layers I-III as most of the nociceptive fibers end there (they include all substance P containing afferent fibers) and tachykinin receptor containing cell bodies are mainly concentrated in these layers. Some recent experiments suggested that also layers V-VI could be very important for noxious stimuli processing in turtle spinal cord (Russo and Hounsgaard, 1994) but it is unclear if this applies to mammals as well. Part of the pain induced sensitisation occurs in the spinal cord and a very similar phenomenon was observed in the spinal cord in vitro (McMahon et al, 1993), firstly in dorsal horn cells and later also in motoneurones. Hence, many pain related events can occur in the spinal cord and, as previously noted, substance P seemed to be a strong candidate for nociceptive transmitter in this region.

TACHYKININS AND PAIN TRANSMISSION

The development of the spinal cord in vitro preparation (Otsuka and Konishi, 1974a) just before the finding by Hökfelt et al (Hökfelt et al, 1975) of substance P immunoreactivity in small spinal fibers opened new possibilities to study nociception. It immediately allowed to show that substance P potentiated the monosynaptic reflex (which is mediated by large fibers, Otsuka and Konishi, 1974b). This is similar to the pain induced facilitation of withdrawal reflex, thus supporting the idea that substance P is the excitatory pain transmitter. Later studies showed that substance P is released mainly upon small diameter fiber stimulation but

not after large myelynated fiber stimulation (Otsuka and Konishi, 1976). More elaborated methods for detection of substance P and tachykinins with the probes covered by antibodies allowed to establish that after noxious stimuli substance P and NKA immunoreactivity increased in the dorsal horn of cat spinal cord in vivo and that NKA immunoreactivity persisted for much longer periods of time (Duggan et al, 1987; Duggan et al, 1990). This suggested that these peptides could have different functional roles. It was found also that substance P and other tachykinins can excite both dorsal horn neurones and motoneurones (Randic an Miletic, 1977, Ireland et al, 1992). Intrathecal injection of substance P and NKA facilitates a spinal nociceptive reflex (Lanueville et al, 1988). The development of tachykinin antagonists allowed to show that these antagonists can act in some cases as analgesic agents and they were reducing part of the pain induced sensitisation (Picard et al, 1993). Taken together all this data indicate that substance P and other tachykinins are strongly involved in nociceptive responses. Nevertheless, the term neurotransmission is very broad and we need more precise definition of what is neurotransmission and neuromodulation and what is known about substance P from this point of view.

SUBSTANCE P AS SENSORY NEUROTRANSMITTER

As pointed out at the beginning of the introduction the mechanism by which a chemical substance can influence synaptic transmission can be very different and these mechanisms can be grouped in two categories:

the first one is classical neurotransmission, when the substance under study is released upon excitation of the presynaptic neurone (this implies that it is also produced in this cell) and evokes early (0.1-100 ms) membrane potential changes on the postsynaptic neurone;

the second one is neuromodulation, which includes changes in the responses induced by other transmitters, release of other transmitters or any other long lasting change in neuronal excitability which cannot be detected directly from membrane potential changes of postsynaptic cell. In this case there is no requirement that the substance is released strictly upon excitation of the presynaptic cell and of an early action.

In both cases the presynaptic cell should produce and store the putative neurotransmitter released upon stimulation; in addition, receptors should be present on the postsynaptic cell together with degradation and/or uptake systems. The first two requirements for substance P are confirmed by immunohistochemical studies (Arvidsson et al, 1990) and, as noted before, tachykinins are released upon thin fiber stimulation (Otsuka and Konishi, 1976, Otsuka and Yoshioka, 1993). The degradation of tachykinins is produced by several specific and non-specific peptidases present in the extracellular space. There is no strong evidence for uptake systems but the degraded products could be taken up (Regoli et al, 1994). In order to establish more precisely the mode of action of substance P and other tachykinins (neurotransmission or neuromodulation or both), first of all the direct action of the substance on neurones should be studied. It was found that substance P depolarises dorsal horn neurones and motoneurones also in the presence of TTX indicating that the action of the peptide is direct (Otsuka and Yanisigawa, 1980; Murase et al, 1982; Ireland et al, 1992; Fisher et al, 1994). These responses show peculiar property, namely in most cases they increase with membrane depolarisation and decrease with hyperpolarisation which is opposite to the fast synaptic potential voltage dependence. Similar properties are also shown by dorsal horn slow potentials evoked by high frequency stimulation (Urban and Randic, 1984). In addition, antibodies to substance P, and the antagonist spantide strongly reduced these slow postsynaptic potentials (Randic et al, 1986). All these data provide strong indication that

substance P (and possibly other tachykinins) act as classical slow neurotransmitters in the dorsal horn of the spinal cord.

Nevertheless, many points remain unclear. Firstly, it was demonstrated that tachykinins can modulate the responses mediated by NMDA sensitive glutamate receptors (Randic et al, 1990; Rusin et al, 1993). In addition, while there is extensive coexistence of substance P and glutamate in small diameter fibers, its functional role is unclear (like in most other cases of coexistence of neurotransmitters). Secondly, a number of substances (GABA, acetylcholine) can be released upon substance P application (an effect indicative of neuromodulation) and part of this release is calcium independent indicating that nonsynaptic mechanisms are involved (Kobayashi et al, 1991; Sakuma et al, 1991). Thirdly, although motoneurones possess functional tachykinin receptors, they receive only a minimum of small substance P containing fibers (the majority of the substance P containing fibers originate from raphe nuclei descending pathways, Arvidsson et al, 1990). Fourthly, there are substance P containing interneurones in the spinal cord (Willis and Coggeshall, 1991), thus some of substance P release after dorsal root stimulation could occur also from these cells. Taken together all this data indicates that tachykinins can have a much more complex role in the spinal cord. One of the possibilities in trying to solve this problem is to study the involvement of tachykinins in postsynaptic potentials of motoneurones during different types of dorsal root fiber stimulation. The choice of motoneurons as test cells is mainly motivated by two facts: firstly, motoneurons are the final step in the reflex arc (which is also modulated by pain); secondly, there are functional tachykinin receptors on motoneurones and these receptors could contribute independently from dorsal horn receptors to pain signalling or/and could have some other function. In order to understand the problem, the synaptic inputs which can produce postsynaptic potentials on motoneurones will be described in more detail.

SPINAL CORD SYNAPTIC INPUTS AND MOTONEURONES

There are several main synaptic circuits in the spinal cord. The fibers which enter the spinal cord can be grouped into several classes (Willis and Coggeshall, 1991).

The fibers of first group originate from the periphery and include sensory fibers from skin, muscles, and internal organs. All of them enter the spinal cord via dorsal root and ganglia. That is the most important group of fibers for the study of pain transmission as they include also small diameter unmyelinated fibers arriving from skin and muscle nociceptors. The dorsal root fibers are classified according to their sensory modality, myelination and thickness (the last two parameters mainly determine the velocity of signal conduction which is also used for the classification). In the simplest classification there are three classes of fibers: large myelinated Aαβ (high conduction velocity, 30-100 m/s; light touch, vibration); small myelinated Aδ (relatively high conduction velocity, 4-30 m/s; joint mechanoceptors, thermal sensation); small unmyelinated C (low signal velocity, 0.5-1 m/s; noxious stimuli) (Willis and Coggeshall, 1991). These fibers can be electrically stimulated selectively as large diameter fibers are more readily activated by short duration shock stimuli (0.1 ms) while small diameter fibers are activated only when longer shock pulses are used (>0.5 ms). These three classes of fibers have different distribution of endings in the spinal cord: Aαβ terminate in the deep dorsal horn plus a small but significant part in the motor nucleus. Conversely, small diameter fibers end mainly in the superficial dorsal horn (including the substantia gelatinosa). Thus, stimulation of dorsal roots should result in synaptic potentials on motoneurones composed of monosynaptic large diameter fiber mediated components and polysynaptic small diameter fiber mediated components. Only small diameter fibers contain substance P immunoreactive varicosities. Coexistence of substance P with glutamate and in some cases with GABA has been demonstrated (De Biasi and Rustioni, 1988, Kachidian et al, 1991).

The second large group of fibers entering the spinal cord arrive from medulla and pons. They originate from several areas (dorsal column nuclei, spinal trigeminal nuclei, reticular formation, raphe nuclei, locus coeruleus). For the present study the most important input is from the raphe nuclei (n. pallidus and n. obscurus) as it terminates in the motor nucleus and make synapses with motoneurones. This group of fibers contains a wide range of cotransmitters (serotonin, TRH, substance P, meth-enkephalin, leu-enkephalin, glutamate, Arvidsson, 1990, Kachidian et al, 1991,). The action of these fibers is implied in the general control of the motoneuron pool during different arousal states. An important projection is also from the locus coeruleus which provides most of the noradrenergic input to motoneurones in rats (Westlund et al, 1982).

The last group of fibers includes all other descending pathways from midbrain, hypothalamus and neocortex. Some of them make direct synapses on motoneurones and the ones from the cortex are believed to be involved in the fine control of conscious movements. There are reports that the projections from midbrain contain tachykinins (Zhuo and Helke, 1993).

Apart from these extrinsic fibers there are local spinal circuitries in which some interneurons show substance P like immunoreactivity. In particular, some cells from the deep dorsal horn layer V and ventral horn contain substance P and could synapse on motoneurones. In addition, it is worthwhile to note that there are at least two classes of inhibitory interneuron which can induce inhibitory postsynaptic potentials on motoneurones (Burke, 1990). The first one is the Renshaw cell in the ventral horn: its peculiar properties is that it can be stimulated by motoneurone axon collaterals and posses extremely high (up to 1000 Hz) firing frequency. The second group of inhibitory neurones is termed Ia and its location is not well determined: it can be directly activated by dorsal root primary afferents.

After this short overview of the spinal synaptic circuitry it is clear that even stimulation of a single pathway can result in complex postsynaptic potentials on motoneurones with several sites of action for substance P and other tachykinins. Several of these pathways (including the descending pathways) are directly or indirectly involved in nociception. Nevertheless, we will be concerned only with dorsal root stimulation evoked events as they are the primary source for noxious stimulus signalling. Therefore, the properties of the postsynaptic potentials on rat spinal motoneurones evoked by dorsal root stimulation will be described.

POSTSYNAPTIC POTENTIALS ON RAT SPINAL MOTONEURONES

In the case of dorsal root stimulation several types of postsynaptic potentials can be evoked on motoneurones depending on the fiber type involved (Fulton and Walton, 1986; Jahr and Yoshioka, 1986; Thompson et al, 1990). Short (0.05-0.1 ms), close to threshold, electrical pulses evoke short, fast EPSP (with delay of several ms and duration of tens of ms). Part of this EPSP is due to direct release of neurotransmitters (in most cases glutamate) from Aαβ dorsal root fibers to motoneurones. The increase in stimulus length (up to 0.5-1 ms) elicits long postsynaptic potentials (up to several seconds) usually with superimposed action potentials. In this case Aδ fibers are also activated. Further increase in stimulus strength will add C fiber activation and will prolong the postsynaptic potential up to 10-20 seconds. It is believed that activation of small diameter fibers (Aδ and C) elicits polysynaptic potentials on motoneurones. All these responses can be also recorded from ventral roots. In this case the response induced by large calibre fiber stimulation only is called monosynaptic reflex as it is analogous to the reflex observed in animals in vivo (Otsuka and Konishi, 1974a).

Repeated stimulation can increase or decrease the postsynaptic potential. For example in the case of Aαβ fiber stimulation at 0.1-5 Hz frequencies there is a decrease in the amplitude

of the response while in the range of 10-50 Hz frequencies it is possible to observe the increase in the amplitude of the postsynaptic potential (Lev-Tov and Pinco, 1992; Pinco and Lev-Tov, 1993). As noxious stimuli are transmitted via small diameter fibers one could expect that postsynaptic potentials evoked by their stimulation should mimic pain induced sensitisation. Indeed, sensitisation in the case of a single neurone is the increase in the number of spikes evoked by a series of afferent stimuli of constant strength. It occurs in the case of the spinal cord at frequencies of 0.5-2 Hz and is termed action potential wind-up (Thompson et al, 1990). It represents the central component of pain induced sensitisation (Woolf, 1983). Although it can be detected in motoneurones with intracellular recordings, several aspects should be mentioned: first of all, it was observed only in minority of the cells; secondly, it was present only in those cells in which small diameter fiber stimulation could evoke postsynaptic potentials longer than 4 s; thirdly, there was clear correlation between the rate of rise of cumulative depolarisation (defined as the rate of rise in membrane potential before each successive stimulus during the train) and the presence of action potential windup; fourthly, there were indications from extracellular recordings that tachykinins are involved in windup and long postsynaptic potentials. The hypothesis that action potential windup is due to modulation of NMDA responses by tachykinins was suggested (Sivilotti et al, 1993).

Another type of protocol used to mimic pain processes is the brief train (about a second) of relatively high frequency C fiber stimulation which induces a long lasting (tens of minutes) increase in spiking activity (this increase could be analogous to a sustained pain sensation). It was demonstrated that only such a kind of stimulation can induce significant increase in substance P release and, in the cat dorsal horn, it was reduced by the NK1 receptor antagonist CP96345 (Koninck and Henry, 1991).

It can be concluded that there are several types of synaptic event related to pain transmission and that the fast postsynaptic potentials (which can be evoked separately by

large diameter fiber stimulation) could be used for understanding the possible involvement of tachykinins in synaptic transmission not related to nociception.

Since in the present thesis the nature of the ionic currents induced by tachykinins is to be addressed, the last section of introduction will discuss the up to date knowledge of the issue.

CURRENTS INDUCED BY TACHYKININS

Tachykinins can change the membrane potential of several types of neurone - dorsal horn cells, motoneurones, ganglion cells. Even in a single cell several different ionic currents can be induced/inhibited (Murase et al, 1989; Shen and North, 1992). In most cases tachykinins can induce a nonselective voltage and/or calcium dependent current (Otsuka and Yoshioka, 1993). It has reversal potential between -30 and 10 mV and is due to increased permeability to both sodium and potassium ions (this conclusion is based on studies with ion substitution, Murase et al, 1989, Spigelman and Puil, 1990, Shen and North, 1992, Inoue et al, 1995). From the point of view of reversal potential and selectivity this current is similar to the ionotropic glutamate receptor induced current. In addition, its calcium dependence has been suggested in some cases as it is strongly reduced in calcium free solution (Murase et al, 1989). Nevertheless, an increase in neurone input resistance has been observed during many responses to substance P indicating block of some current or/and strong voltage dependence of the induced current (Minota et al, 1981; Fisher et al, 1994). There are several currents which tachykinins can suppress: M currents (Jan and Jan, 1982); the current carried through inward (anomalous) rectifier channels (Yamaguchi et al, 1990); the current carried through potassium selective channels (according to the reversal potential of the current) and blocked by millimolar concentrations of barium (Fisher and Nistri, 1993). In the last two cases the blocked current is supposed to be at least partially active at the resting potential and

therefore this current contributes to the leakage current. The block of such a current will reduce potassium permeability of the cell membrane and the cell membrane potential will move towards sodium reversal potential (as the proportion of sodium current will increase), that is it will depolarise. A similar effect (increase in input resistance and depolarisation at the same time) could happen also in the case of activation a nonselective cationic current activated by depolarisation (Samir and Andrade, 1996). In addition, there are reports that tachykinins can facilitate or inhibit calcium currents (Shapiro and Hille, 1993). These complex effects obscure the detection of the currents underlying the responses. In the case of motoneurones it has been shown that substance P shares the intracellular signalling pathway with TRH, another putative peptidergic neurotransmitter, and mainly inhibits a barium sensitive potassium current (presumably leakage current). Therefore, the responses have a similar, unusual voltage dependence (decrease with hyperpolarisation) as in most dorsal horn cells (Fisher and Nistri, 1993). Nevertheless, the study has been done with intracellular recording which does not allow good voltage clamp crucial for establishing the nature of ionic currents and their kinetics.

AIMS OF THE PRESENT THESIS

The main purpose of this thesis was to establish the role of tachykinin receptors (with emphasis on the receptors of motoneurones) in synaptic transmission from dorsal root fibres to motoneurones. As described in the introduction there are data which support the hypothesis that substance P (the principal endogenous mammalian tachykinin) is a slow neurotransmitter in the dorsal horn of the spinal cord. Nevertheless, substance P could contribute to synaptic transmission in many ways and the role of tachykinin receptors on motoneurones is still obscure as discussed in the introduction. The project of this thesis

aimed at clarifying the way in which substance P contributed to different synaptic responses on motoneurones during dorsal root stimulation. To this end highly specific antagonists for tachykinin receptor subtypes and intracellular recording techniques were employed. Intracellular recordings are important for detecting slow and small responses typical of responses presumably mediated by substance P in the dorsal horn of the rat spinal cord (Murase et al, 1982). The data on the contribution of each receptort type to fast and slow synaptic potentials revealed by specific antagonists could help to understand better the role of substance P and tachykinins in sensory transmission in the spinal cord. The aims of the project were pursued with the following steps:

1) first, by application of highly specific agonists to detect tachykinin receptor subtypes effects on motoneurones directly or indirectly. Binding and in situ hybridisation studies have demonstrated the presence of NK1 and NK3 receptors in rat spinal cord (Beresford et al, 1992) but no comprehensive functional studies have been performed at the spinal cord level. It was needed to know precisely the functional receptor subtypes in order to choose the antagonist correctly;

2) second, to test the antagonist potency in the neonatal rat spinal cord since species differences could exist;

3) third, to investigate the effects of the potent tachykinin antagonists on responses to different fiber stimulation, or to stimulus trains as the slow time course of tachykinin induced responses could be important for synaptic input integration.

In addition, it was attempted to find out the ionic basis of substance P induced currents on motoneurones by employing patch-clamp whole-cell technique (using in this case hypoglossal motoneurones in brainstem slices).

METHODS

For the present experiments two kind of preparations were used: the isolated spinal cord (Otsuka and Konishi, 1974a) and the brainstem slice. The second preparation was used for characterisation of the currents underlying substance P responses for the following reasons: firstly, it is a well described preparation of motoneurones in slices (Bayliss et al, 1994), with hypoglossal motoneuron basic properties similar to the ones of spinal motoneurones; secondly, they possess relatively high density of tachykinin receptors and substance P containing fibers (Helke et al, 1985, Connaughton et al, 1986), thirdly, there are technical difficulties in obtaining spinal cord slices of the age of 5-13 days (this is the age which was used for the isolated spinal cord preparation) with viable motoneurones. Slices are more suitable for using whole cell patch clamp techniques for more accurate voltage clamp experiments and more precise current characterisation.

Spinal cord preparation

Experiments were performed using neonatal Wistar rats (5-13 day old, both sexes). Under urethane anaesthesia the rat was decapitated and the thorax was quickly cut and opened. After rinsing with ice cold salt solution the rat was fixed by pins to the silicon bottom of a petri dish. The spinal cord was removed and dissected out with its roots attached in the continuous presence of ice cold oxygynated with 95% O_2 and 5% CO_2 salt solution in the petri dish. The composition of solution in both cases was (mM): NaCl, 113; KCl, 4.5; $MgCl_2$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; pH 7.4. The spinal cord was subsequently transferred to a recording chamber and continuously superfused (7ml/min) at room temperature (19-24°C) with oxygenated salt solution of the same composition as the

one used for the dissection. The dorsal and ventral roots were sucked by small (~0.5 mm tip diameter) pipettes which were used for root stimulation and/or recording. The viability of the preparation was routinely checked by the presence of ventral root response (usually of 0.5-1.0 mV amplitude) to stimulation of dorsal roots (by 0.1 ms electric pulse of 3-5 V amplitude). The spinal cord was kept for at least 1 hour before the start of the experiments in a recording chamber for recovery from dissection. Homologous L4 or/and L5 dorsal and ventral roots were stimulated via suction electrodes. Motoneurones functionally identified by their antidromic spike were impaled with 3 M KCl or 2 M potassium methylsulphate or 5 M potassium acetate microelectrodes (the resistance of the electrode was in the range 30-100 MΩ) under discontinuous current clamp conditions (2 kHz sampling rate). In a few cases the bridge mode of current clamp was used.

Several types of dorsal root stimulation were used to elicit postsynaptic potentials. The pulses of 0.1 ms duration were used to estimate the threshold for synaptic potentials due to activation of Aαβ fibers (detected as the appearance of fast postsynaptic potentials, with 50-150 ms duration, < 10 ms delay and < 10 mV amplitude). The threshold was usually in the range of 0.5-1.0 V. The stimuli of 0.1 ms were applied with > 15 s interval in order to avoid the decrease in amplitude of the responses (Lev-Tov and Pinco, 1992). The longer pulses (1 ms) were used to elicit high threshold slow postsynaptic potentials (duration > 4s, amplitude- >20 mV with at least one early spike). All properties of these postsynaptic potentials (duration, ability to summate. spike number) and the stimulus amplitude (5 - 10 times the threshold detected with 0.1 ms pulses) suggest that C fibers were also activated. In this case the minimal interval between stimuli was 90 s. One ms pulses of the same amplitude were used to study windup and the associated cumulative depolarisation. In this case the responses were elicited by 1 Hz trains of 20-30s duration (Thompson et al 1990; Sivilotti et al 1993, the appearance of the response is shown in the figure) and they were accompanied

by long lasting (>15 s) afterdepolarisations. These trains, applied at a minimum interval of 3 min, elicited reproducible responses which were averaged from at least two tests. The following parameters of cumulative depolarisations evoked by such trains were measured (see Fig.1 , the same letters are used in the figure and in the text):

a) the upstroke was measured as the difference between the baseline and membrane potential before the second stimulus;

b) the peak was measured as the differences of membrane potential before the train (the baseline) and before the last stimulus in the train;

c) the decay area of afterdepolarisation was measured during the 20 s starting 1 s after the end of the train;

d) the rate of rise was measured (starting from the 3rd stimulus in the train) as the rate of increase in membrane potential before each successive stimulus (Sivilotti et al., 1993);

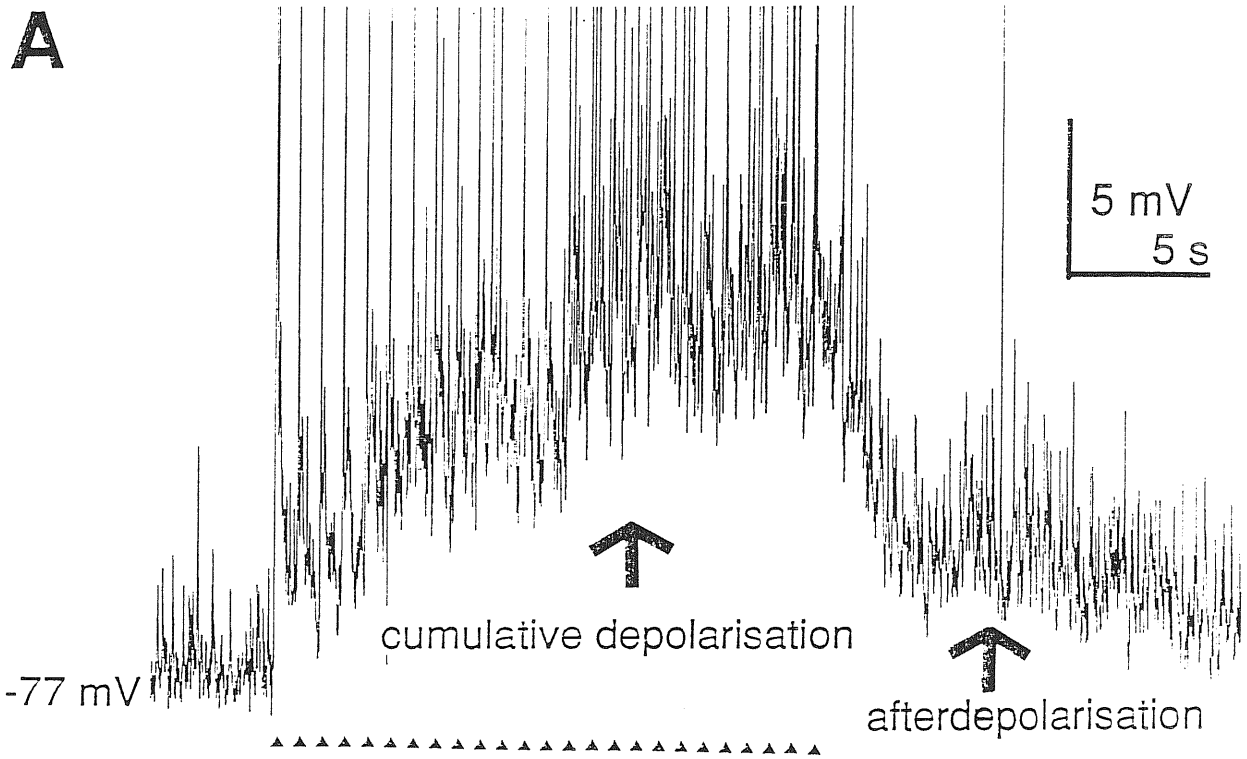
e and f) the area of postsynaptic potential during the train was measured as the area between the membrane potential before the stimulus in the train (the first, e in Fig. 1, or the last, f in Fig.1) and the potential attained during 1 s after stimulus.

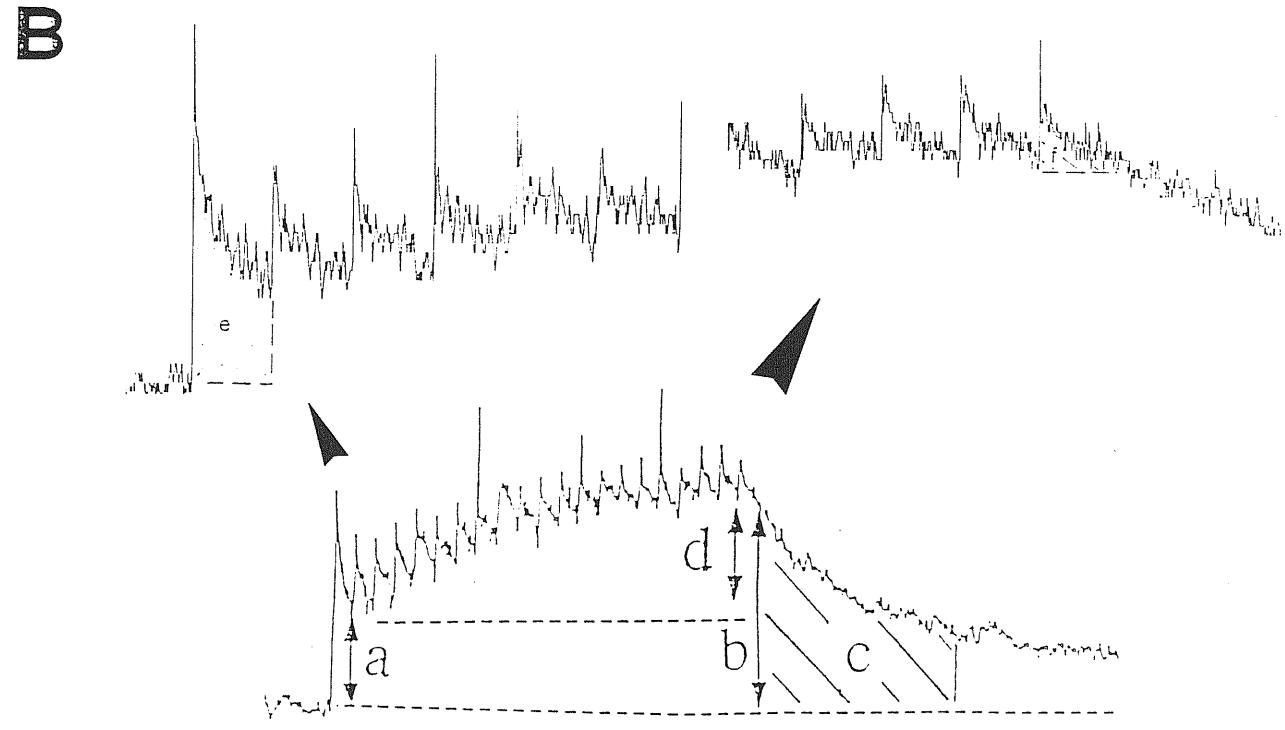
g) the area of cumulative depolarisation was measured from baseline to membrane potential developed during the stimulus train starting from the 1st stimulus;

All parameters were measured from averaged computer traces (the number of trials used for averaging was: >9 for fast postsynaptic potentials, >2 for the slow ones and 2-3 for cumulative depolarisations).

The signal to be stored in computer files was sampled at 5 kHz in the case of fast postsynaptic potentials, at 0.5 kHz in the case of long postsynaptic potentials and at 100 Hz in the case of cumulative depolarisation. Data were analysed with a commercial software (pCLAMP 6; Axon Instruments) and are presented as mean ± SEM.

Fig. 1. A. An example of action potential windup and cumulative depolarisation induced by 1 ms 7 V 1 Hz stimulus train. The stimuli were delivered at the times indicated by triangles under the trace. Upward deflections represent spikes (note the increase in frequency at the end of the train). Resting membrane potential was -77 mV. B. The scheme of the measurement of cumulative depolarisation parameters (see text for their definitions). a- upstroke, b- peak, c- area of afterdepolarisation, d- index comprising rate of rise multiplied by the duration of the train (the actual value of the rate of rise is obtained by dividing d) by the duration of the train), e and f- the area of postsynaptic potential during the train (e- of the first one and f- of the last one, see the insets on the top).


A
5 mV
5 s
cumulative depolarisation
-77 mV
afterdepolarisation


B
e
d
a
b
c

Brainstem slice preparation

Neonatal Wistar rats (3-8 days old) were used. The head of animal was cut and placed in ice cold oxygenated salt solution of the following composition (mM): NaCl, 130; KCl, 3; $MgCl_2$, 2; $CaCl_2$, 2; NaH_2PO_4, 1.25; $NaHCO_3$, 26; glucose, 10; pH 7.4. The skull was opened and the cerebral hemispheres were quickly removed. After cutting neck muscles and underlying cranial bones the brainstem with cerebellum was placed into another dish with cold salt solution of the same composition. After removal of cerebellum the brainstem was pinned by its ponto-cerebellar surface facing downward to the agar block and transverse slices of 150-200 microns were cut in ice cold oxygenated salt solution. Before experiments the slices were incubated for at least 1 hour at 35°C. For experiments the slices were placed in a recording chamber under a nylon mesh (for fixing them into position) and superfused at room temperature (24°C) with a solution of the same composition as the one used for preparing the slices. The pipettes for patching were fabricated from glass capillaries (1.5 mm o.d.) and filled with solution of the following composition (mM): potassium gluconate, 122.5; NaCl, 9; KCl, 17.5; EGTA, 0.5; ATP (potassium salt), 2; GTP (sodium salt), 0.5 (omitted in some experiments, see results); HEPES, 10: $MgCl_2$, 1; pH was adjusted to 7.2 by KOH. The resistance of electrodes after filling was 3-6 MΩ. The hypoglossal motoneurones for whole-cell patch clamp recording were identified visually by immersion optics (Zeiss, Germany) as the largest cells in the hypoglossal nucleus (most of the cells in the nucleus are motoneurones, Viana et al, 1990). The recordings were done using L/M-PCA (List-electronic, Darmstadt, Germany) amplifier. The analysis of the currents were done with commercial software (pClamp 6; Axon Instruments, U.S.A.).

Drugs

To avoid neuronal desensitisation due to repeated applications of tachykinin receptor agonists, these compounds were applied usually for 5 min at 30-45 min intervals. The antagonists were applied for at least 15 min before the tests were done. All test compounds were bath applied. The following drugs were used:

1) the tachykinin agonists (all agonists were kindly donated by Dr. C. Maggi, Menarini Pharmaceuticals, Florence, Italy):

substance P methyl ester (SPMeO) for NK1 receptor,

[βAla^8]NKA$_{4\text{-}10}$ for NK2 receptor,

senktide or [MePhe^7]neurokinin B (NKB) for NK3 ;

2) the nonpeptide tachykinin receptor antagonists

RP 67580 ((3aR, 7aR)-7,7-diphenyl-2-[1-imino-2(2-methoxyphenyl)ethyl] perhydroisoindol-4-one, kindly donated by Dr. C. Maggi, Menarini Pharmaceuticals, Florence, Italy), a selective NK1 receptor antagonist;

SR 140333 ((S)-1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenyl)-piperidin-3-yl]ethyl]-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride, generously donated by Dr. X. Emonds-Alt, Sanofi Recherche, Montpellier, France), a selective blocker of the NK1 receptor (Emonds-Alt et al, 1993);

CP 99994 (dihydrochloride salt of (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine), kindly donated by Pfizer Inc, CT, U.S.A, a selective blocker of NK_1 receptor (Watling and Crause, 1993);

SR 48968 ((*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl]benzamide, generously donated by Dr. X. Emonds-Alt, Sanofi Recherche, Montpellier, France), a selective NK2 receptor blocker;

3) glutamate receptor antagonists (purchased from Tocris Cookson, England):

non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX);

NMDA receptor antagonists R-aminophosphonovalerate (APV; 25 μM) and 3-((RS)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 20 μM);

4) substance P and part of substance P methyl ester was purchased from Sigma, Milano, Italy.

RESULTS

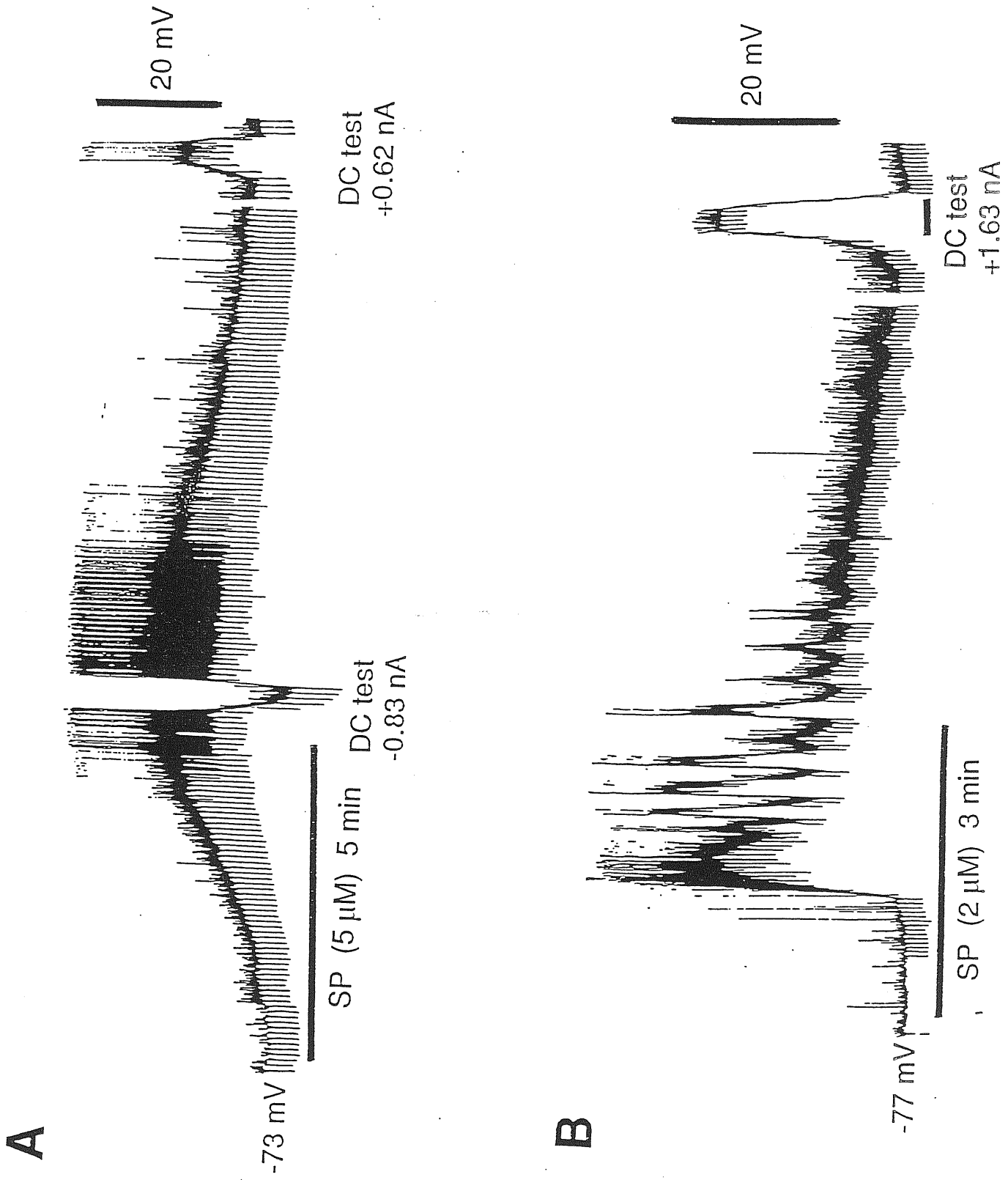
Part I. Spinal cord preparation

The experiments were done on 140 motoneurones with -74 ± 0.5 mV resting membrane potential and 21 ± 2 MΩ input resistance. Antidromic spike (80 ± 2 mV, in a random sample of 20 cells) could be evoked in all of them by ventral root stimulation indicating that they were motoneurones. No distinction between alfa and gamma motoneurones could be made (at this age they are at the stage of differentiation). The parameters of motoneurones are close to the ones previously reported with intracellular recordings (Fulton and Walton, 1986).

Agonist tests

In order to study tachykinin receptor involvement in synaptic transmission on motoneurones we need to know first which types of tachykinin receptors are functional in the spinal cord of the neonatal rat. This can be tested by application of specific agonists. Substance P can act on all three subtypes of tachykinin receptor (Maggi et al, 1993). It is thus important to test it for comparison with other more specific agonists. Fig. 2A shows the typical response elicited by application of substance P (5 μM) to a motoneurone (resting potential -73 mV) in control solution. The response was a slow depolarisation which persisted for several minutes after washout. The substance P (2 μM)-induced depolarisation

Fig. 2. Current clamp recordings from two different motoneurones at resting membrane potential responding to 5 (A) or 2 μM (B) substance P. Input resistance was monitored by injecting 500-ms current pulses (-0.2 nA for A and -0.4 nA for B) through microelectrode at 0.2 Hz. Application of substance P (see horizontal bars) elicited sustained responses characterized by a slow onset depolarization associated with increased neuronal activity detected as spontaneous postsynaptic potentials (seen as thickened baseline) and an increase in input resistance, as indicated by larger voltage responses to injected current pulses. In A, substance P elicited tonic firing of action potentials (truncated by frequency response of pen recorder) superimposed on gradually developing depolarisation, while, in B, it evoked a more rapid depolarisation with membrane oscillations and burst firing. Amplitude of steady state DC current injected during DC tests is indicated below corresponding chart record. Note that slow depolarisation elicited by DC current evoked no action potential firing.

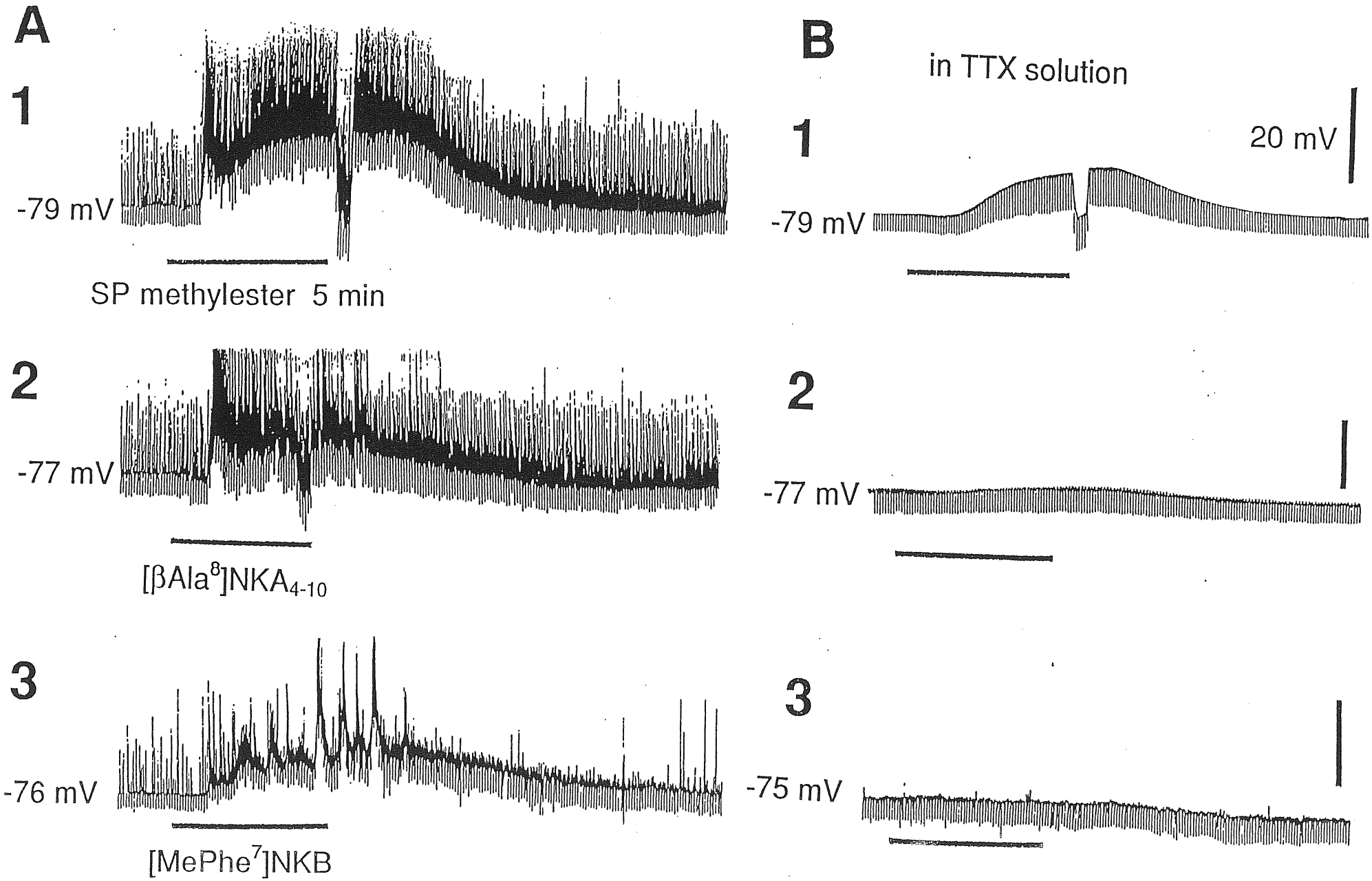

A
20 mV
DC test
+0.62 nA
DC test
-0.83 nA
-73 mV
SP (5 μM) 5 min
B
20 mV
DC test
+1.63 nA
-77 mV
SP (2 μM) 3 min

(16 ± 3 mV; n = 5) was clearly associated with increase in synaptic activity (see thickening of baseline record), sustained action potential firing and an apparent increase in cell resistance (25 ± 10%), as indicated by the larger amplitude of electrotonic potentials (downward deflections) evoked by constant current pulses. Passive depolarisation of the neurone via intracellular current injection to a comparable level of membrane potential did not change the cell resistance as shown by the DC test performed after recovery from drug application (Fig. 2A): this finding indicates that the resistance observed during the drug application was caused by substance P and not by mere rectification resulting from membrane depolarisation. A DC test was also performed at the peak of the response to substance P to repolarise the cell to its resting value: this procedure showed that at resting potential the peptide-induced resistance increase was attenuated. Since rectification was not responsible for resistance changes, it follows that the modulation of cell resistance by substance P was itself a voltage dependent phenomenon. Although the duration of drug responses and long washout intervals did not allow construction of dose-response curves, the action of substance P was found to be dose dependent in 0.1-10 μM range, in accordance with earlier data obtained with extracellular recording (Ireland et al, 1992). In 2/21 cells, substance P elicited a response like the one of Fig. 2B in which bursts of spikes superimposed on slow oscillations of membrane potential accompanied a long-lasting depolarisation and resistance increase (these phenomena were not caused by membrane depolarisation as shown by the DC test). The action of substance P on motoneurones is proposed to be due to activation of receptors found presynaptically on interneurones and postsynaptically on motoneurones (these components of the response differ in their sensitivity to TTX, Ireland et al, 1992). In TTX (1 μM) solution which largely isolated postsynaptic effects, 2 μM substance P induced a 19 ± 10 mV

depolarisation with 50 ± 10% rise in input resistance (n = 4). The action of substance P thus appears to be chiefly mediated postsynaptically.

The action of SPMeO (highly specific agonist for NK1 receptor), [Ala]NKA (NK2 receptor agonist) or [MePhe]NKB (NK3 receptor agonist) on different rat motoneurones in control or TTX solution is shown in Fig. 3 A,B. On all cells tested in control solution, the selective NK1 receptor agonist SPMeO (200 nM) elicited slow depolarisation (25 ± 4 mV; n = 4) associated with an increase in cell resistance (15 ± 5%) and, usually, tonic action potential firing (Fig. 3A1) although in two neurones slow membrane potential oscillations with burst firing appeared against a background of phasic action potential discharge. In TTX solution, the action of SPMeO persisted in all cells tested (Fig. 3B1; 14 ± 3 mV depolarisation with 46 ± 10% resistance increase, n = 4), a result suggestive of a predominantly postsynaptic action. The effects of this substance were thus very similar to those usually induced by substance P. The action of the selective NK3 receptor agonist [MePhe]NKB (200 nM) on a cell in control solution is displayed in Fig. 3A3. In all cells tested, this compound elicited a slow, steady depolarisation (11 ± 0.5 mV, n = 17) with an apparent increase in input resistance (15 ± 3%) typically accompanied by voltage oscillations with superimposed spike bursts without background repetitive firing. This type of response was not found after application of SPMeO to the same cell even when the depolarisation amplitude was similar to the value attained with [MePhe]NKB. In TTX solution the effect was usually lost (see Fig. 3B3) since on average, [MePhe]NKB induced depolarisations (19 ± 2 mV) and increased cell resistance (by 50 ± 15%) in 3/11 cells only, indicating a predominantly presynaptic action. The response to the selective NK2 receptor agonist [Ala]NKA (200 nM) in control solution is shown in Fig. 3A2. The depolarisation (10 ± 2 mV; n=23) was accompanied by a 15 ± 3% rise in input resistance and often by irregular

Fig.3. Current clamp recordings from two different motoneurones (tracings 1 and 2 are from the same cell) at resting membrane potential responding to SPMeO (1), [Ala}NKA (2) or [MePhe]NKB (3) in control (A) or TTX solution (B), Input resistance was monitored as for Fig. 2 (amplitude of current pulses was -0.3 nA). Application of agonists (see horizontal bars) in control solution elicited delayed and sustained depolarisations associated with increase in input resistance and enhanced synaptic activity. Depolarisation induced by SPMeO (Fig. A1) was smooth in progression and was associated with tonic action potential firing. In TTX solution, response induced by this compound. (Fig. B1) largely persisted. Note that manual repolarization during peak of depolarisation (current amplitude was -0.42 nA) revealed that observed increase in input resistance was not merely a voltage dependent phenomenon (Fig. A1, B1). In control solution, [Ala]NKA induced depolarisation (Fig. A2) did not develop smoothly, and phasic firing of action potentials occurred. Manual repolarisation (current amplitude was -0.34 nA) demonstrated that increase in cell resistance was not only a voltage dependent effect. In presence of TTX (Fig. B2), [Ala]NKA induced a much shallower depolarisation that progressed smoothly with minimal change in input resistance. In control solution, [MePhe]NKB induced a depolarisation (Fig. A3) associated with a bursting pattern of firing superimposed on membrane potential oscillations. In TTX solution, this agonist elicited no response (Fig. B3). All agonist concentrations were 200 nM.


A
1
-79 mV
SP methylester 5 min
2
-77 mV
[βAla8]NKA4-10
3
-76 mV
[MePhe7]NKB
B
in TTX solution
1
20 mV
-79 mV
2
-77 mV
3
-75 mV

firing patterns although bursts typical of NK3 receptor activation were absent (Fig. 3A2). In TTX solution [Ala]NKA elicited slight depolarisations (see Fig. 3B2) which on average was 3 ± 0.5 mV associated with no evident change in input resistance (6/11 cells). This substance, therefore, appeared to act mainly at presynaptic sites. An interesting feature of the action of [Ala]NKA and [MePhe]NKB (Fig. 3A) or senktide (not shown) was the rise in cell resistance during the responses in control solution, the effect absent in TTX containing solution. It suggests that these agonists induced via TTX sensitive mechanisms a release (from interneurones) of endogenous neurotransmitter(s) which postsynaptically increased motoneuronal input resistance.

Fig. 4A shows a case where the NK_3 agonist [MePhe]NKB did induce a depolarising response even in TTX solution. This depolarisation progressed smoothly like in control solution except that the bursts where absent. Moreover the response to another NK_3 agonist senktide had similar time course and it also persisted in TTX solution in the same cell (Fig. 4B). It indicates that the presence of the responses to NK_3 agonists in TTX solution in some cells was not artifactual and its time course was more dependent on the activated receptor subtype.

It was previously mentioned that responses to NK3 agonists were accompanied by slow voltage oscillations. It is worth noting that agonists for all three subclasses of tachykinin receptor (though NK1 and NK2 only in some cases) could evoke oscillations as shown in Fig. 5. Nevertheless, only NK3 receptor agonist responses were always accompanied by regular oscillations. These oscillations could not be reproduced by simple membrane depolarisation and TTX always blocked them (see example in Fig. 6) indicating that they were network dependent phenomena.

These experiments demonstrated that:

first, all types of tachykinin receptor are functional in the neonatal rat spinal cord;

second, two of them- NK1 and NK3- are present on motoneurones (though the NK3 one is present in a minority of cells only);

third, the response properties (duration, presence and shape of slow membrane potential oscillations and/or bursts) were mainly determined by activation of a certain receptor type;

fourth, substance P was most likely acting via NK1 receptors (as the responses to substance P and to the NK1 receptor agonist SPMeO were similar and always persisted in TTX solution). Experiments with specific NK1 receptor antagonists were then performed.

Antagonist tests

The first antagonist to be tested was RP 67580. It was chosen for two reasons: first, it is a nonpeptide compound and should not have stability problems; second, it was believed to be more specific for the rat subclass of NK1 receptor type (Barr and Watson, 1993). The concentration of RP 67580 was 1-2 μM, since it was found to be sufficient to saturate receptors (Barr and Watson, 1993) and in no cell such a concentration had any direct effect on motoneurone membrane. Nevertheless, the results of experiments performed in our lab could not find a consistent antagonism by RP 67580 on NK_1 receptor mediated responses. Substance P was tested first. In control solution substance P (1-2 μM, lowest concentration to induce a reliable response) elicited a sustained depolarisation (10 ± 2 mV; n = 5) associated with a rise in input resistance (35 ± 12%) and repetitive firing (comprising bursting in the example of Fig. 7A). No antagonism of substance P effects was observed following application of RP 67580 for up to 1 hour in 4 out of 5 cells.

Fig. 4. Current clamp recordings of the responses to two different NK_3 receptor agonists, [$MePhe^7$]NKB and senktide, in the same motoneurone. The left side panels show responses in the control solution and the right-hand ones show the responses in the presence of TTX (1 μM). The upper traces show the responses to [$MePhe^7$]NKB while the lower traces show the responses to senktide. The drugs were applied for 5 min (indicated by horizontal bars). The resting potential is shown before each trace. The upward deflections in the traces in control solution represent spontaneous postsynaptic potentials and spikes truncated by recorder frequency response. Note a large increase in spontaneous activity (including spikes) during the responses in control solution and the absence of any spontaneous activity during the responses in TTX solution while the smooth depolarisation persisted. The downward deflections represent the electrotonic potentials induced by -0.2 nA current pulses and were used to monitor the increase in input resistance during responses. Note that both agonists produced very similar responses both in control and TTX solutions. DC tests (to return membrane potential to the resting value in the middle of the response) were performed during all responses. Agonist concentration was 200 nM.

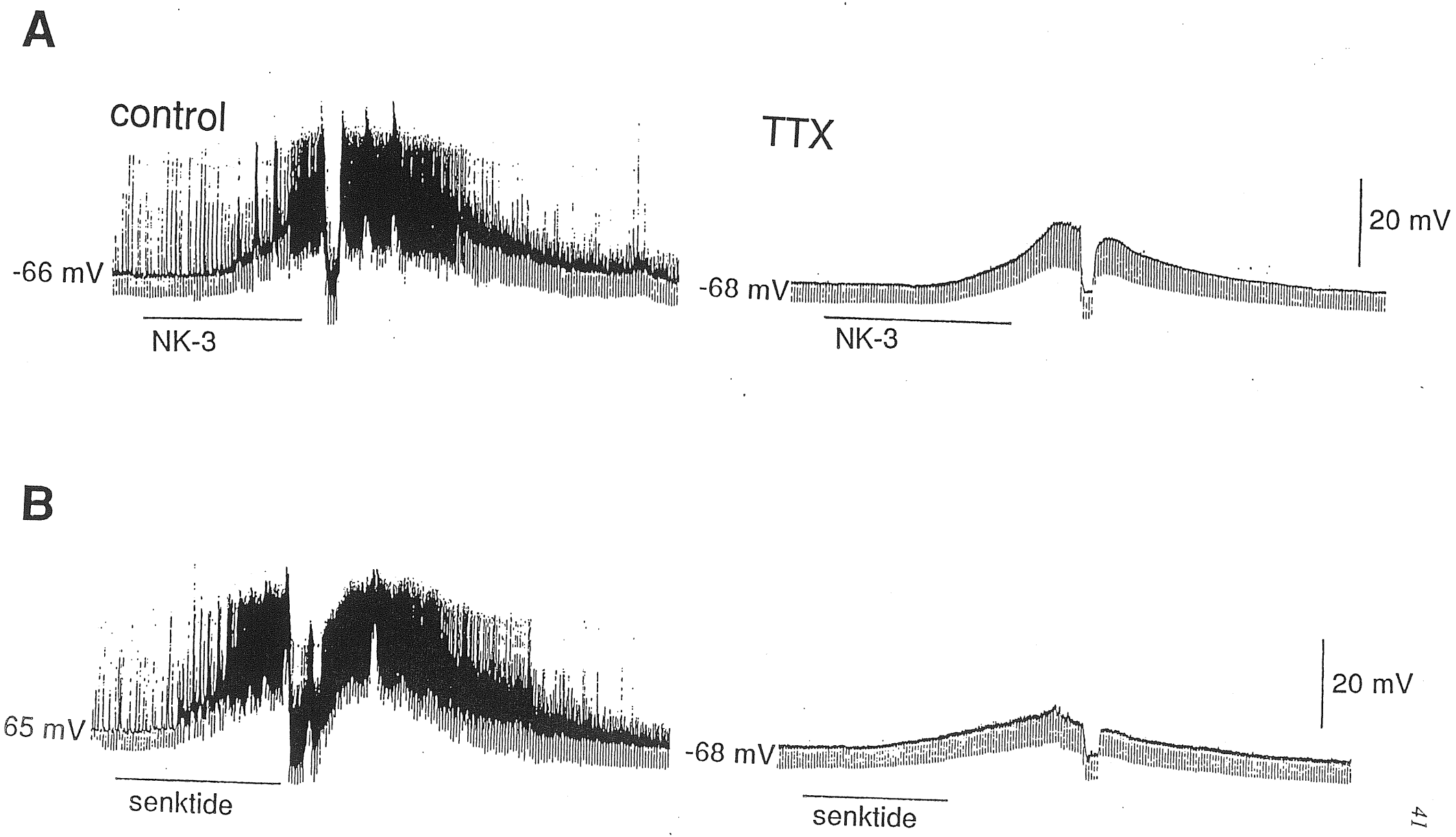

A
control
TTX
20 mV
-66 mV
-68 mV
NK-3
NK-3
B
65 mV
20 mV
-68 mV
senktide
senktide

Fig. 5. Examples of bursting activity induced by agonists to three tachykinin receptors: SPMeO (for NK_1 receptors, the upper trace), [Ala^8]NKA_{4-10} (for NK_2 receptors, the middle trace), and [$MePhe^7$]NKB (for NK_3 receptors, the bottom trace). The traces represent membrane potential recordings (the resting potential is indicated before each trace). The drugs (all at 200 nM concentration) were applied for the time indicated by horizontal bars. For further details see Fig. 2 legend.

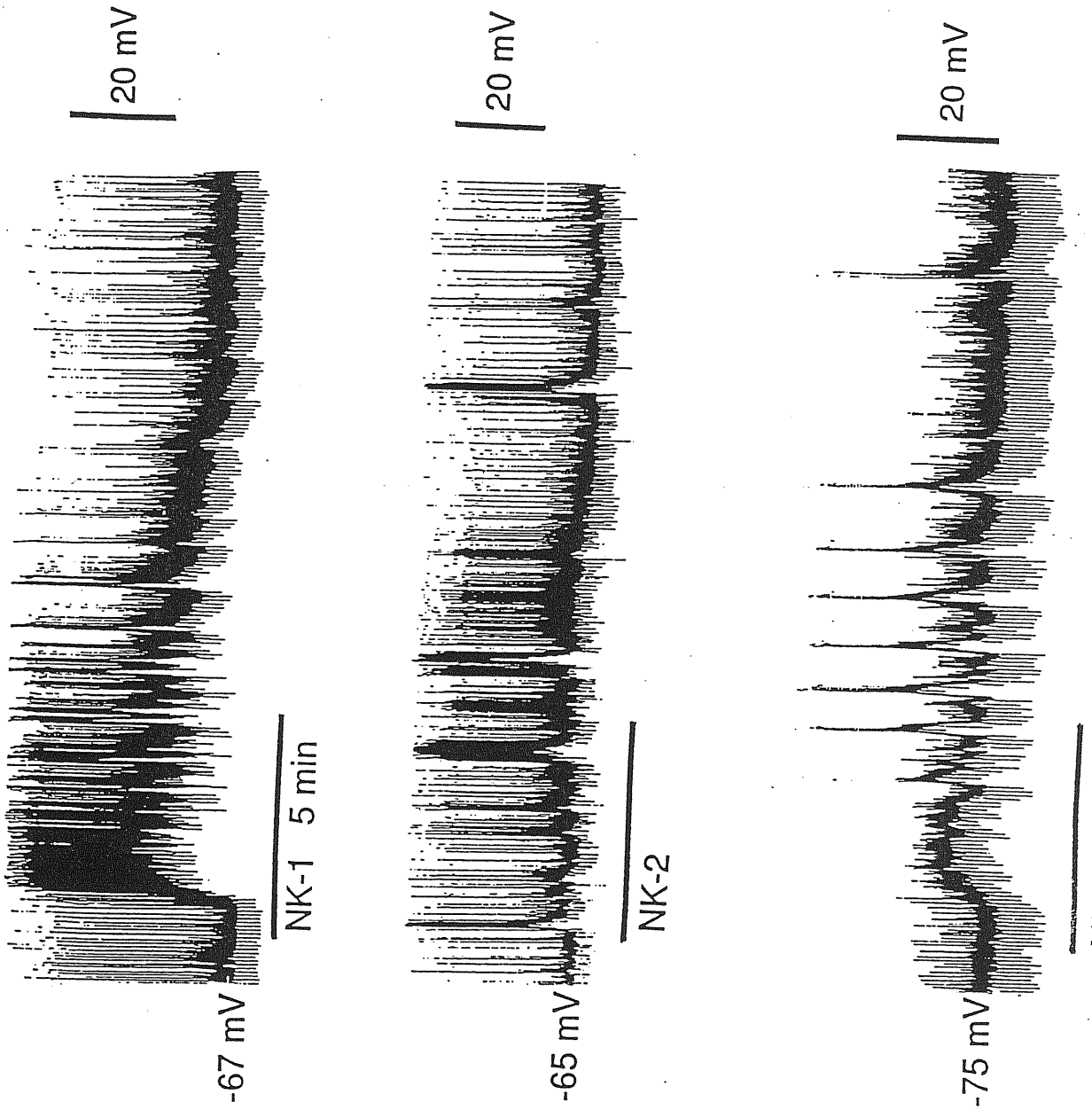

20 mV
-67 mV
NK-1 5 min
20 mV
-65 mV
NK-2
20 mV
-75 mV
NK-3

Fig. 6. Membrane potential oscillations induced by the tachykinin receptor agonist senktide are not reproduced by simple membrane potential depolarisation and they are blocked by TTX (1 μM) application. The tracings show membrane potential changes of the cell under current clamp in control solution (the upper trace) and in TTX solution (the lower trace) after application of senktide (200 nM, 5 min, see horizontal bars). The input resistance was continously monitored by injection of -0.2 nA current pulses (downward deflections in the tracings). In the control solution application of the drug induced membrane potential oscillations with associated large increase in spontaneous activity and spikes (upward deflections in the trace, spikes are truncated by recorder frequency response). In the upper trace the membrane of the cell was depolarised before the response (DC test) by injection of positive current. Note that no oscillations were observed. In the lower trace the spontaneous activity was blocked by TTX and the response to senktide developed smoothly.

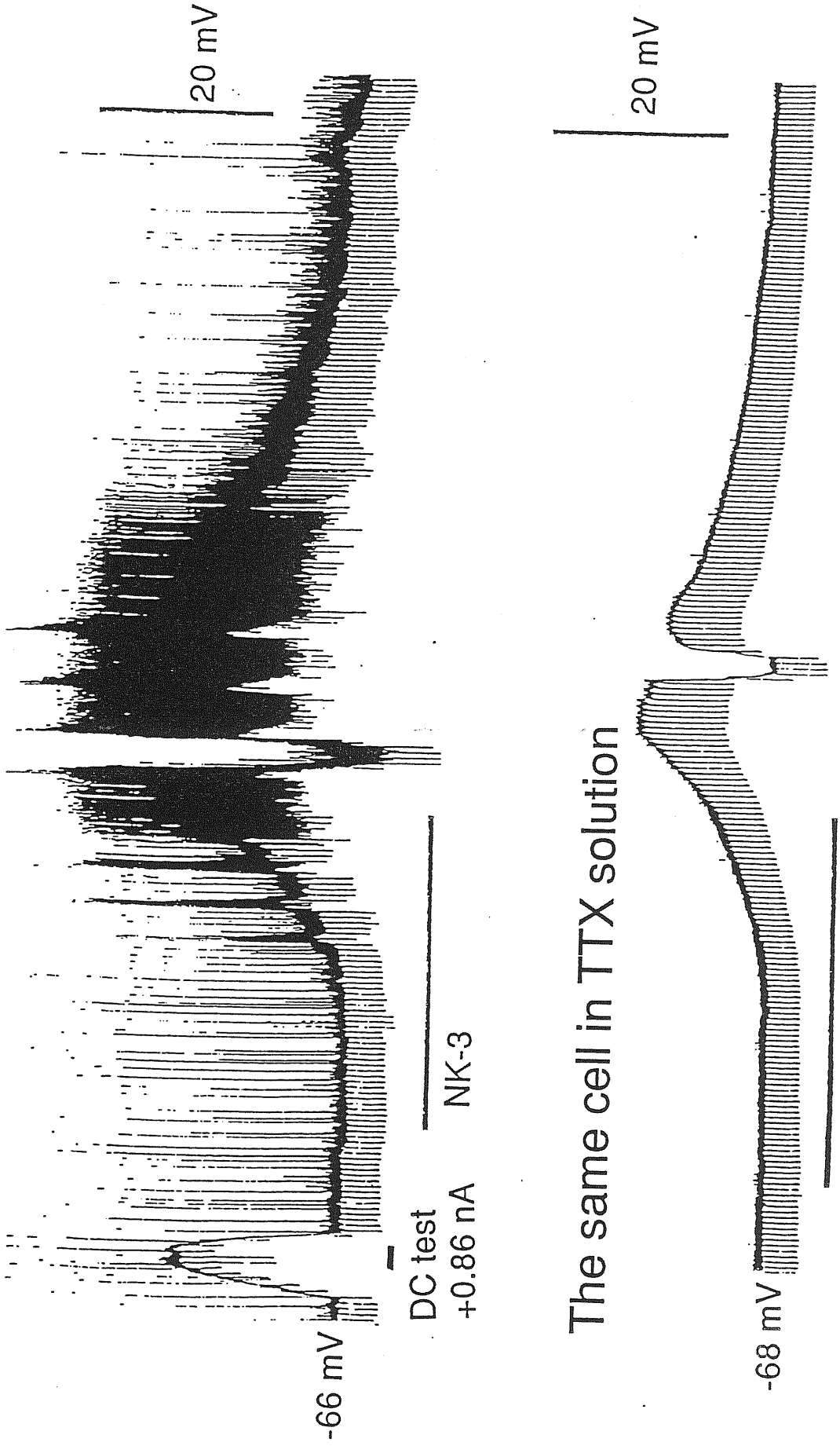

20 mV
-66 mV
DC test
+0.86 nA
NK-3
The same cell in TTX solution
20 mV
-68 mV
NK-3

Fig. 7. Effects of SP on membrane potential and input resistance before or after application of RP 67580 (1 μM). Cell resting potential (mV) is shown before each trace. SP (2 μM) was applied for a time indicated by horizontal bar below the tracings either in control solution (A) or in TTX solution (B). Upward deflections comprise spontaneous synaptic potentials and spikes (clipped by frequency response of the recorder) often arising from the top of bursting activity induced by SP application. Downward deflections are hyperpolarising electrotonic potentials elicited by constant current pulses (-0.2 nA; 500 ms) injected via microelectrode. Note a large enhancement of depolarisation evoked by substance P that, however, recovers after 45-min washout of RP 67580 (bottom trace). (B) Responses recorded in the presence of 1 μM TTX using the same protocol as in (A) except that the injected current amplitude is -0.1 nA. Note lack of effect of RP 67580.

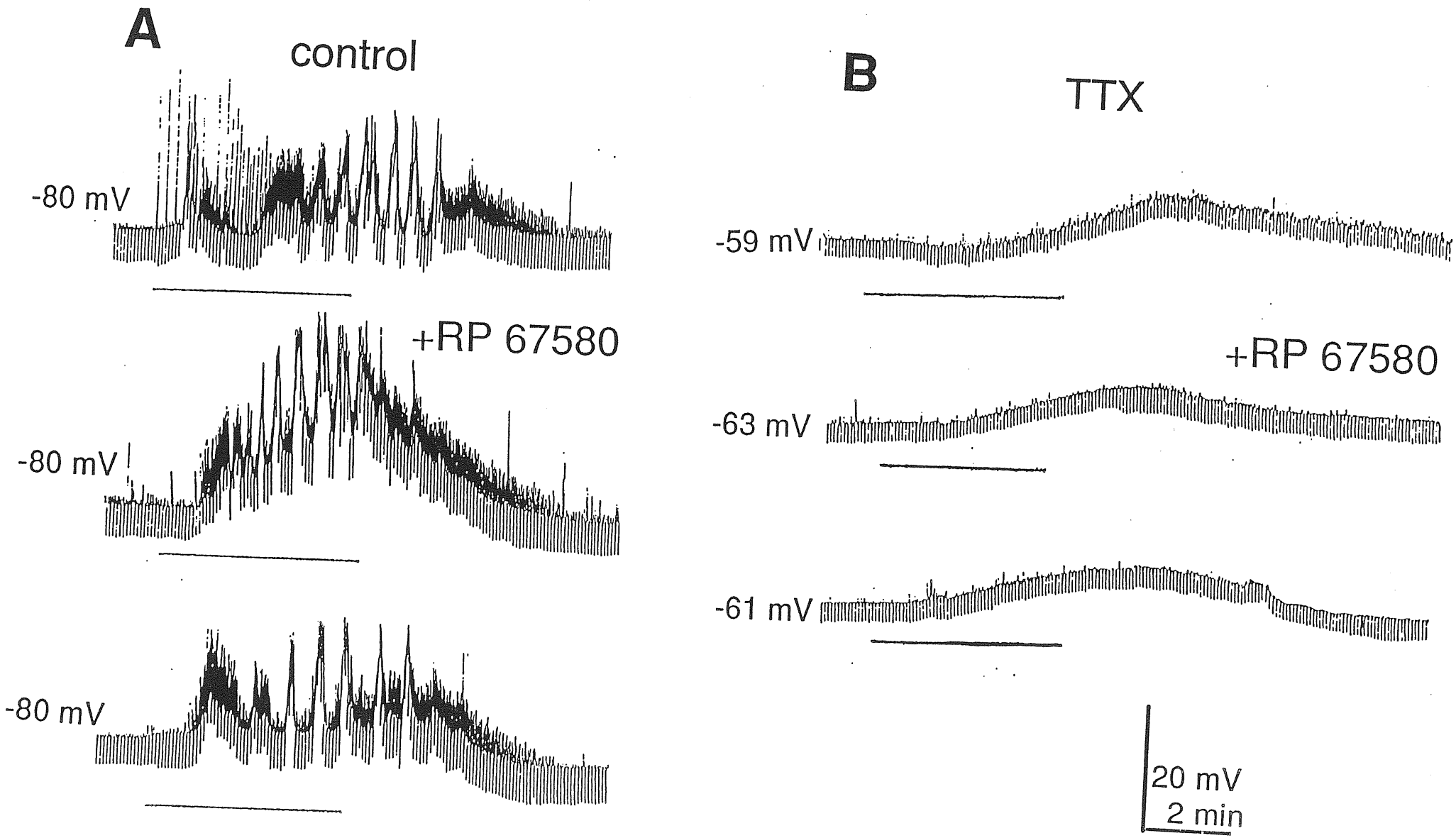
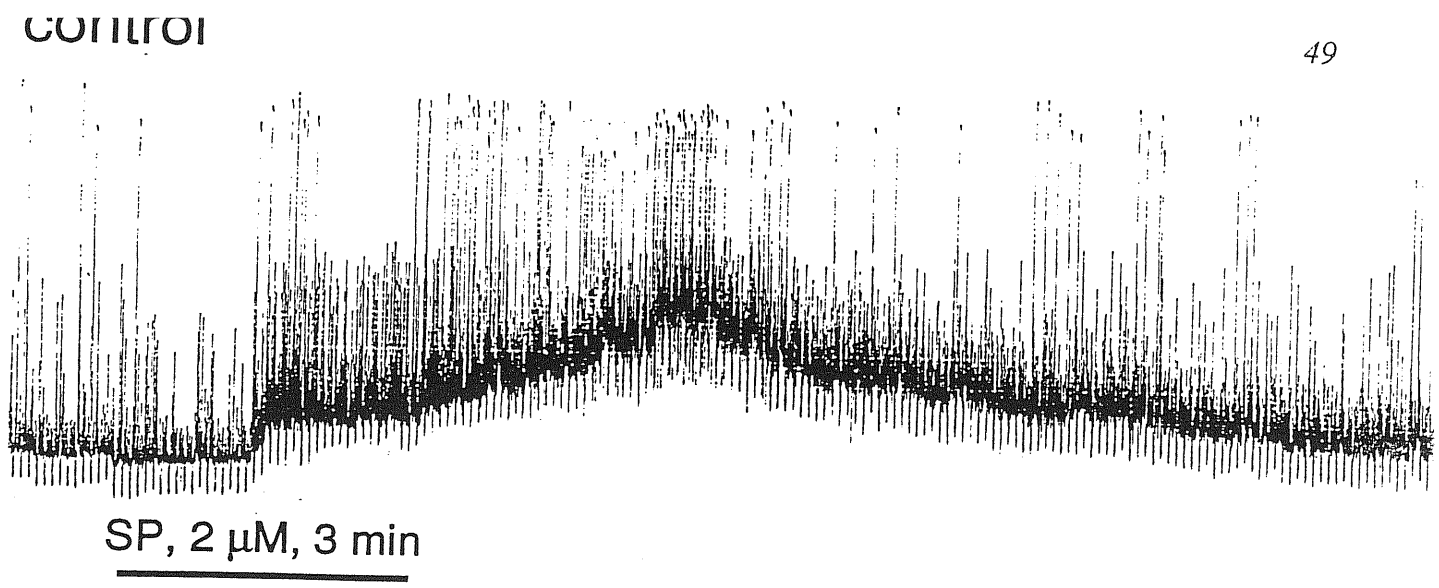
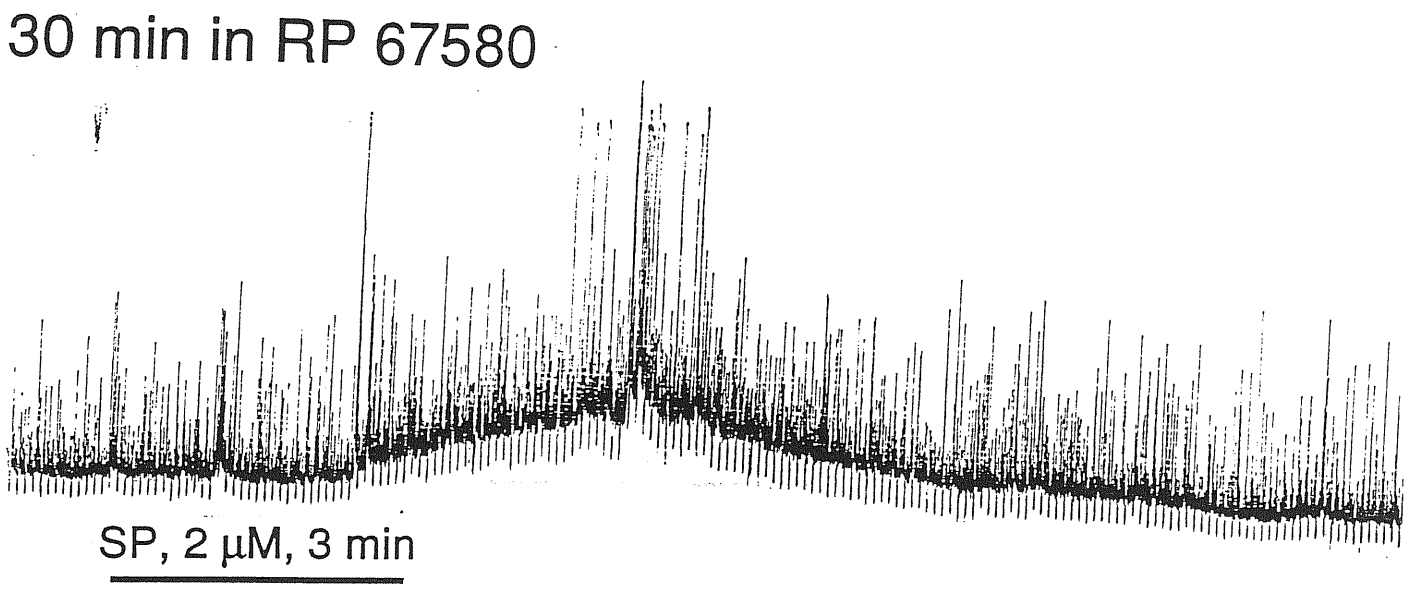
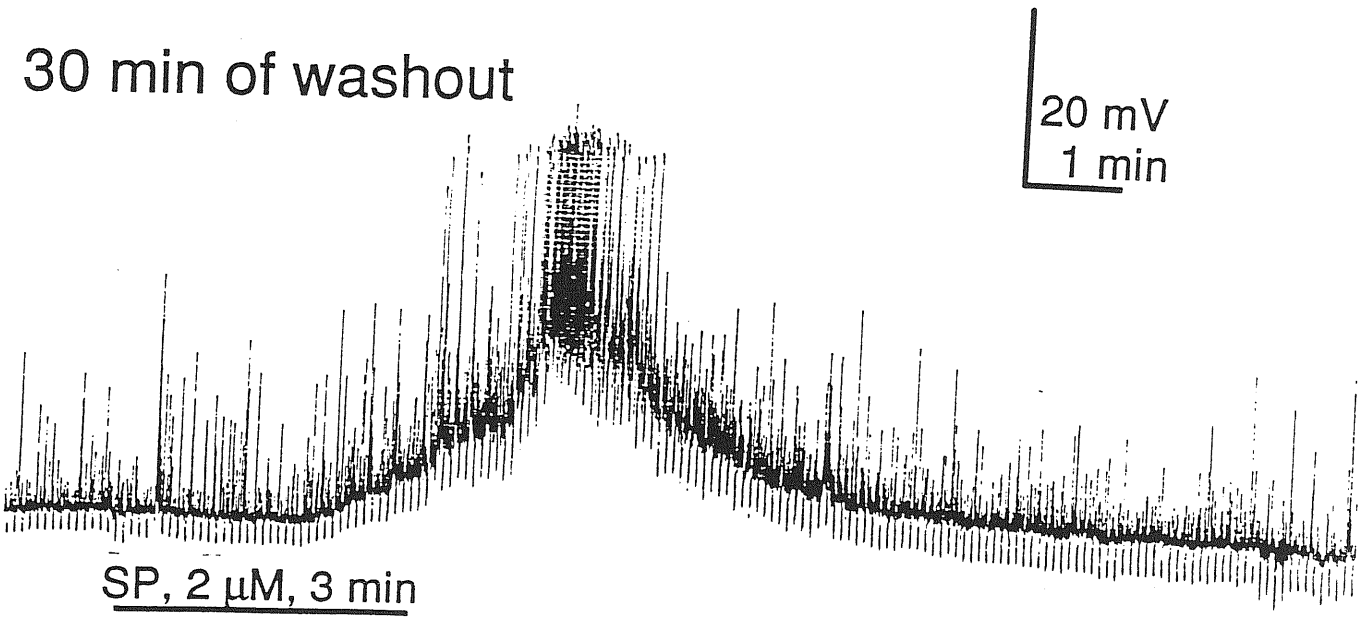

A
control
-80 mV
+RP 67580
-80 mV
-80 mV
B
TTX
-59 mV
+RP 67580
-63 mV
-61 mV
20 mV
2 min

Fig. 8. Example of the response to substance P depressed by RP 67580 (1 μM). The motoneurone was under current clamp (the traces show membrane potential changes) and the resting potential was - 72 mV in all cases. The application of substance P (see horizontal bar) in control solution induced a smooth depolarisation accompanied by increase in spontaneous activity (upward deflections) and in input resistance (increase in electrotonic potentials induced by -0.2 nA current pulses and represented in tracings as downward deflections). In the presence of RP 67580 the smooth depolarisation and increase in spontaneous activity was clearly reduced (the middle trace) and it partially recovered after 30 min washout (the lower trace).


control
SP, 2 µM, 3 min


30 min in RP 67580
SP, 2 µM, 3 min


30 min of washout
20 mV
1 min
SP, 2 µM, 3 min

In one other cell the depolarisation and resistance increase produced by substance P was unchanged (4 mV depolarisation in control and 4 mV in 1 μM RP 67580 solution) while in one cell only it was depressed (Fig. 8). When TTX (1 μM) was applied to prevent indirect actions of substance P, RP 67580 did not alter the depolarisation amplitude (14 ± 4 mV vs 14 ± 4 mV) or resistance increase (100 ± 25 vs 135 ± 30 %) induced by this peptide (see also Fig. 7B). As these results could be explained by an RP 67580-resistant action of substance P via non NK1 receptors the specific agonist SPMeO was tested in TTX solution (in order to minimize the complex neuronal network effects). In this case SPMeO (100-200 nM; n=6) in TTX solution elicited responses (12 ± 2 mV depolarisation and 26 ± 9% resistance rise) comparable to the ones evoked by 1-2 μM substance P. Following application of RP 67580 to these six cells bathed in TTX solution, no significant changes were obseved either for the SPMeO depolarisation or resistance increase (12 ± 3 mV and 47 ± 24%, respectively, for 6 responses in the presence of RP 67580) compared with the ones observed before RP 67580 administration. On four other neurons the inactive isomer RP 68581 (1 μM) produced no change in the substance P mediated responses.

In spite of mainly negative results with agonist responses the effects of RP 67580 on synaptic transmission were also tested. As the effect could be fiber specific, three patterns of stimulation were used. First, low threshold fast postsynaptic potentials (presumably due to Aαβ fiber activation induced by 0.1 ms 1-2 V pulses) were affected neither in amplitude nor in decay by RP 67580 (1-2 μM) in all cells tested (n = 5; an example is shown in Fig. 9A). Second, 1 ms 5-10 V stimulus induced high threshold slow postsynaptic potentials (4-20 s duration, presumably due to C fibers activity, see introduction),were unchanged (115 ± 13%; see also Fig. 9B) in amplitude and duration in 8/9 cells exposed to RP 67580. Third, long lasting (for up to 90 s) depolarisations induced by 10 Hz 5 s trains of

the same strength of stimuli (used to simulate noxious stimuli, Koninck and Henry, 1991), were also unaffected in 7/8 cells (112 ± 11% of control area of depolarisation during the first 60 s after the train, see an example in Fig. 9C).

Since RP 675680 was usually ineffective as NK1 antagonist, other nonpeptide antagonists were tested. CP 99994, a recently developed NK1 antagonist, is a modified version of the prototype antagonist CP 96345 (Watling and Krause, 1993). This substance (at 1 μM concentration) also appeared to be ineffective against NK1 receptor agonist (substance P methylester) induced responses in the spinal cord in 3 out of 4 trials (an example is given in Fig. 10). Conversely, the novel nonpeptide NK1 receptor antagonist SR 140333 (Emonds-Alt et al, 1993) was very potent and quite specific. Fig. 11A shows that SR 140333 (1 μM; 15-40 min application) completely blocked the action of SPMeO (200 nM) but spared that of the NK3 agonist senktide (200 nM; Fig. 11B). The action of SPMeO was tested at various intervals after the start of application of SR 140333 in order to establish the minimum time needed to produce full antagonism: this was between 10-15 min. On average SR 140333 (1 μM; 15 min application) fully abolished responses to SPMeO (n=7; a phenomenon usually reversible after 30-45 min washout) while it reduced responses to the NK2 agonist [Ala]NKA to 60 ± 30 % (n = 2; tracings not shown) and left unaffected those to the NK3 agonist senktide (n = 2, Fig. 11B). These results indicate that SR 140333 was a reasonably selective and highly potent NK1 receptor antagonist on the neonatal rat spinal cord preparation. It should be noted that SR 140333 was acting on all NK1 receptors throughout the spinal cord, as it blocked motoneurone depolarisation (mainly a postsynaptic NK1 receptor effect) as well as the increase in background spontaneous synaptic activity (mainly a presynaptic NK1 receptor effect).

At the time when the experiments were done only NK2 receptor antagonists were available in addition to NK1 receptor antagonists.

Fig. 9. Synaptic potentials evoked by electrical stimulation of dorsal root fibers in control solution and in the presence of RP 67580. Left column shows responses in control solution and right-hand one shows responses in the presence of RP 67580 (2 μM); resting membrane potential values are indicated below each trace. (A) Average trace (n = 5) displaying low threshold fast postsynaptic potential (preceded by hyperpolarising electrotonic potential induced by -0.1 nA current pulse) evoked by 0.1 ms stimulus applied to the dorsal root (see arrowhead). (B) Average traces (n = 5) of high threshold responses elicited by 1 ms dorsal root stimulation (see arrowhead). Spikes were cancelled by averaging procedure. (C) Single tracings displaying slow responses with superimposed spike activity (clipped by the pen recorder) induced by stimulus train (indicated by horizontal bar; 100 pulses; 1 ms long; 10 Hz). After interruption in the trace (2 min on the left and 5 min on the right), recovery of resting membrane potential is shown. (A), (B), and (C) are from different cells. Calibration bars: 20 m, 30 ms in (A); 50 mV, 1.5 s in (B); 30 mV, 20 s in (C).

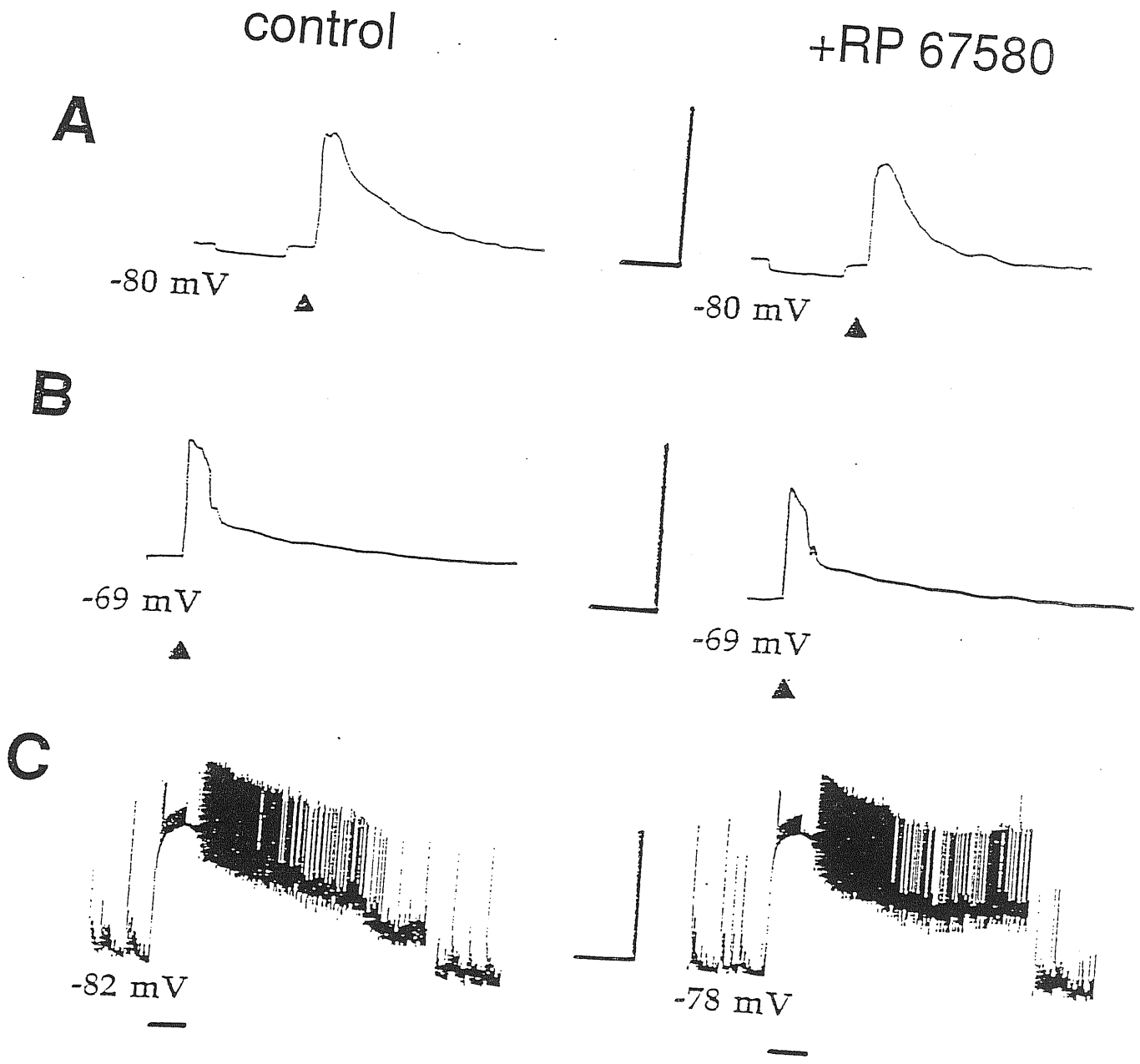

control
+RP 67580
A
-80 mV
-80 mV
B
-69 mV
-69 mV
C
-82 mV
-78 mV

Fig. 10. Example of the response to NK_1 receptor agonist SPMeO which was increased in the presence of the NK_1 receptor antagonist CP 99994 (1 μM). The neuron was under current clamp (the tracing shows membrane potential) and the resting membrane potential for both tracings was -84 mV. The response to the agonist in the control solution was a small smooth depolarisation accompanied by a large increase in spontaneous activity (upward deflections; the downward deflections represent electrotonic potentials induced by -0.3 nA current pulses, used to monitor the input resistance). After superfusion for 10 min with CP 99994 the response to SPMeO (the membrane potential depolarisation and increase of spontaneous activity) was larger than in control conditions.

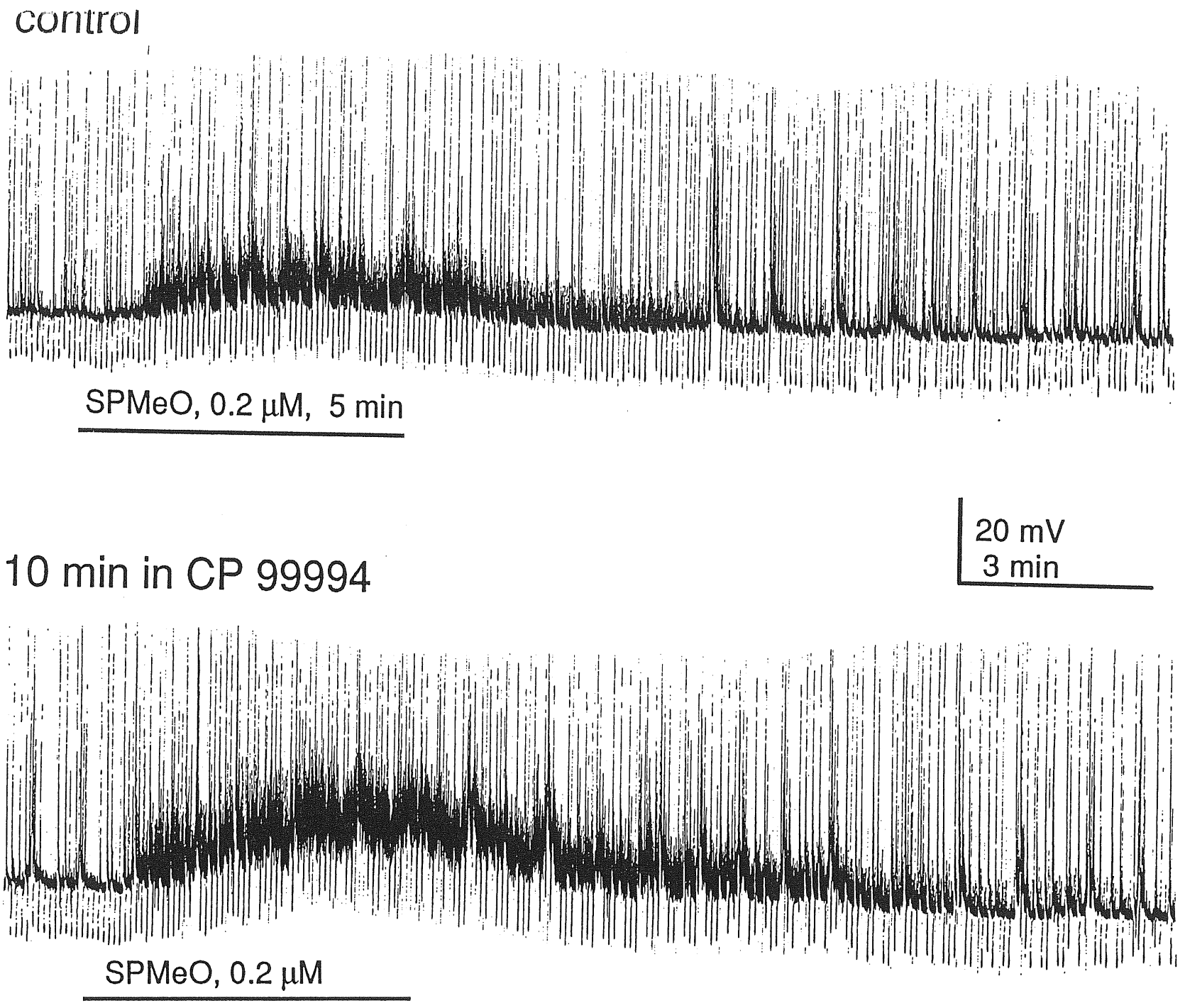
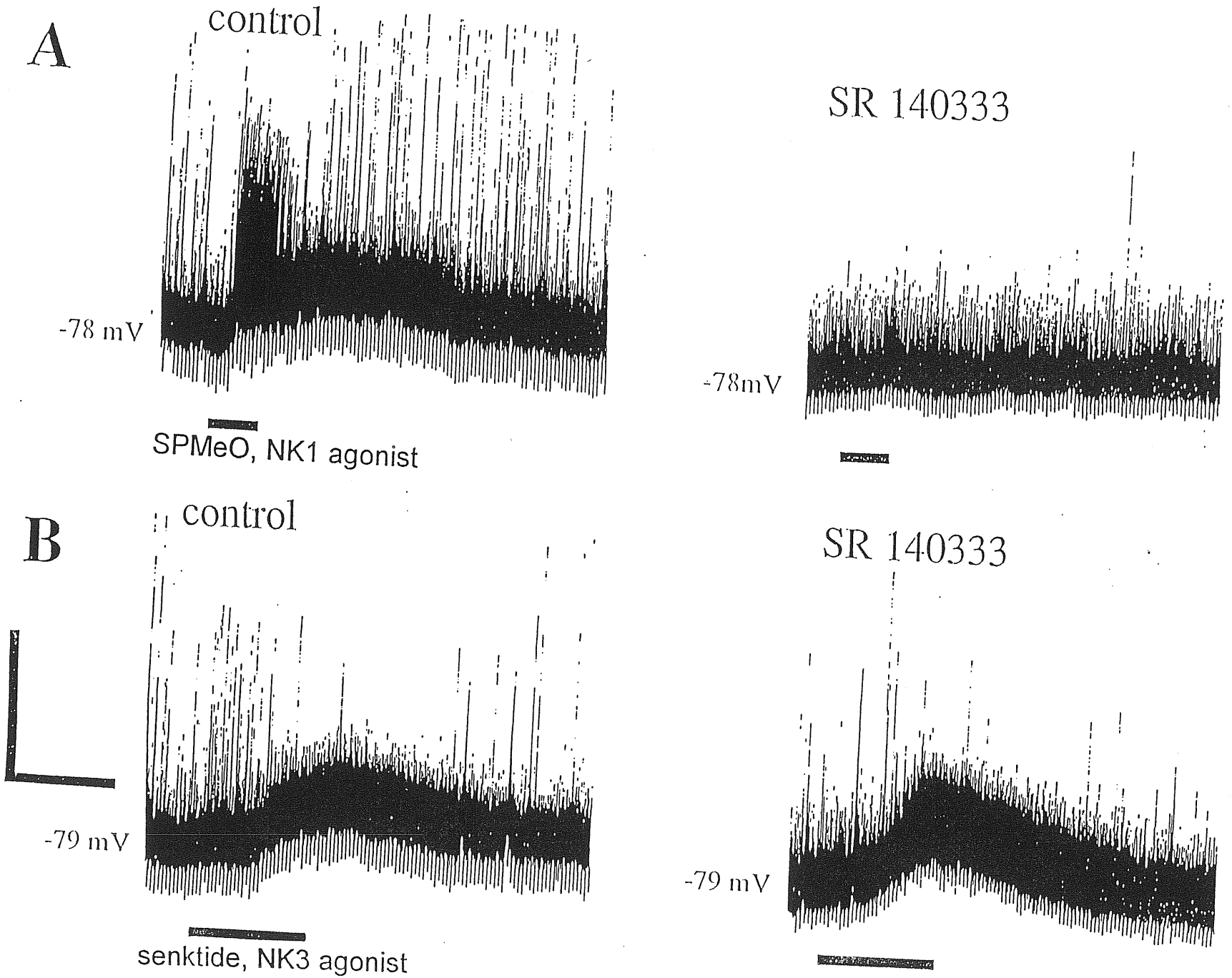

control
SPMeO, 0.2 μM, 5 min
20 mV
3 min
10 min in CP 99994
SPMeO, 0.2 μM

Fig. 11. Effects of the NK_1 receptor antagonist SR 140333 (1 μM) on responses induced by SPMeO (an agonist at NK_1 receptors) and senktide (an agonist at NK_3 receptors). A and B are from the same cell. Agonists (200 nM) were applied for times indicated by the horizontal bars before or after 20 min application of SR 48968. Traces represent membrane potentials with resting values indicated beside the traces. The responses (whenever present) were developing as smooth depolarisations of membrane potential accompanied by increase in spontaneous activity. Spontaneous activity is displayed as thick baseline because individual events are not resolved at this speed; rapid upward transients represent synaptic potentials reaching spike treshold; spike peak clipped by recorder frequency response. Calibration bars: 15 mV, 5 min.


A
control
SR 140333
-78 mV
-78mV
SPMeO, NK1 agonist
B
control
SR 140333
-79 mV
-79 mV
senktide, NK3 agonist

We tested SR 48968 which was developed by the same group (Emonds-Alt et al, 1992) and had similar chemical structure. Therefore, it was likely to be useful in the neonatal rat spinal cord: our experiments confirmed that suggestion. Figure 12A shows that SR 48968 (500 nM; 20 min application) reduced the amplitude of the depolarisation produced by the NK2 agonist [βAla]NKA (200 nM) from 14 to 9 mV and decreased the number of voltage oscillations present during such a depolarisation. Conversely, the same concentration of SR 48968 slightly enhanced the depolarising responses induced by the specific NK1 agonist SPMeO (200 nM; Fig. 12B). On average the action of the NK2 agonist was reduced by 50 ± 20% (n = 5) while that of SPMeO was not significantly increased (19 ± 7%; n=2). Responses evoked by the NK3 agonist [MePhe]NKB (200 nM) were completely unaffected by SR 48968 (500 nM; n = 2, data not shown). It can be concluded that SR 48968 is quite specific for NK2 receptors. Even though it was not suppressing completely the NK2 receptor agonist induced responses, it should be adequate to reveal NK2 receptor contribution to synaptically induced responses.

The effects of NK1 and NK2 antagonists on postsynaptic potentials on motoneurones

Three types of synaptically induced responses were studied before and after applying antagonists (see methods): fast postsynaptic potentials, long postsynaptic potentials and cumulative depolarisations (induced by 1 Hz trains). Experiments with glutamate receptor (ionotropic non-NMDA and NMDA) antagonists were also performed. This was needed for two reasons. First, to test a possible interaction between tachykinin receptors and NMDA receptor mediated transmission: an issue of importance in the view of the data on modulation of NMDA agonists induced responses in spinal neurones (Rusin et al, 1993). Second, as there are several possible sources of endogenous tachykinins in the spinal cord (primary thin

diameter sensory fibers, interneurones), the full block of iontropic glutamate receptors could help to find out if there is any direct action of tachykinins released from primary afferent fibers on motoneurones. The experiments provided a negative answer to this question (see Fig.13). In control solution 1 ms stimulus produced a long lasting (>10 s) postsynaptic potential of 35 mV amplitude (Fig. 13A, thin trace). It was fully blocked by the co-application of 10 μM of CNQX, a non-NMDA receptor antagonist, and 25 μM of APV, NMDA receptor antagonist, see Fig. 13A, thick trace, which is in agreement with previous studies (Jahr and Yoshioka, 1986; Ziskind-Conhaim, 1990; Konnerth et al, 1990; King et al, 1992; inhibitory postsynaptic potentials evoked directly by afferent fibers are smaller and visible in minority of cells only, Jiang et al, 1990). Recovery was obtained after 30 min washout. Increasing the strength up to five-fold or application of 1 Hz train, which produced in the control solution a cumulative depolarisation of 17 mV peak amplitude (Fig. 13B, thin trace) failed to reinstate these responses (Fig. 13B, thick trace, amplitude < 2 mV). It indicated that intact glutamatergic transmission was an absolute requirement for recording slow postsynaptic potentials from motoneurones. Thus, the first group of experiments with tachykinin antagonists was done in the absence of glutamate receptor antagonism. In control solution 1.6 V 0.1 ms stimulus (in this motoneuron 0.1 ms stimulus threshold for response to dorsal root stimulation was 0.8 V) induced a complex, low threshold, fast (<300 ms duration) postsynaptic potential (Fig. 14A). It was composed of the first peak (~10 ms delay) which presumably corresponded to the monosynaptic response and much slower polysynaptic components (with 20 - 70 ms delays) which formed multiple peaks of higher amplitude (low threshold slow component). All characteristics (the delay of the first peak, duration and the stimulus strength) suggest that only Aαβ fibers were activated (Thompson et al, 1990).

Fig. 12. Effects of the NK_2 receptor antagonist SR 48968 (0.5 μM) on responses to [βAla^8]NKA_{4-10} or SPMeO of motoneurones. Agonists were applied for times indicated by the horizontal bars before or after 20 min application of SR 48968. Traces represent membrane potentials (resting values are indicated beside the traces) with spontaneous activity (upward deflections) and hyperpolarising electrotonic potentials (downward deflections; elicited by 500 ms, -0.2 nA current pulses). Note oscillatory activity induced by [βAla^8]NKA_{4-10} (a preferential agonist at NK_2 receptors) while SPMeO (a preferential agonist at NK_1 receptors) elicited a larger, slowly developing depolarisation. In both examples the agonist concentration used was 200 nM. A and B are from different cells. Calibrations for A and B are 15 mV and 3.5 min.

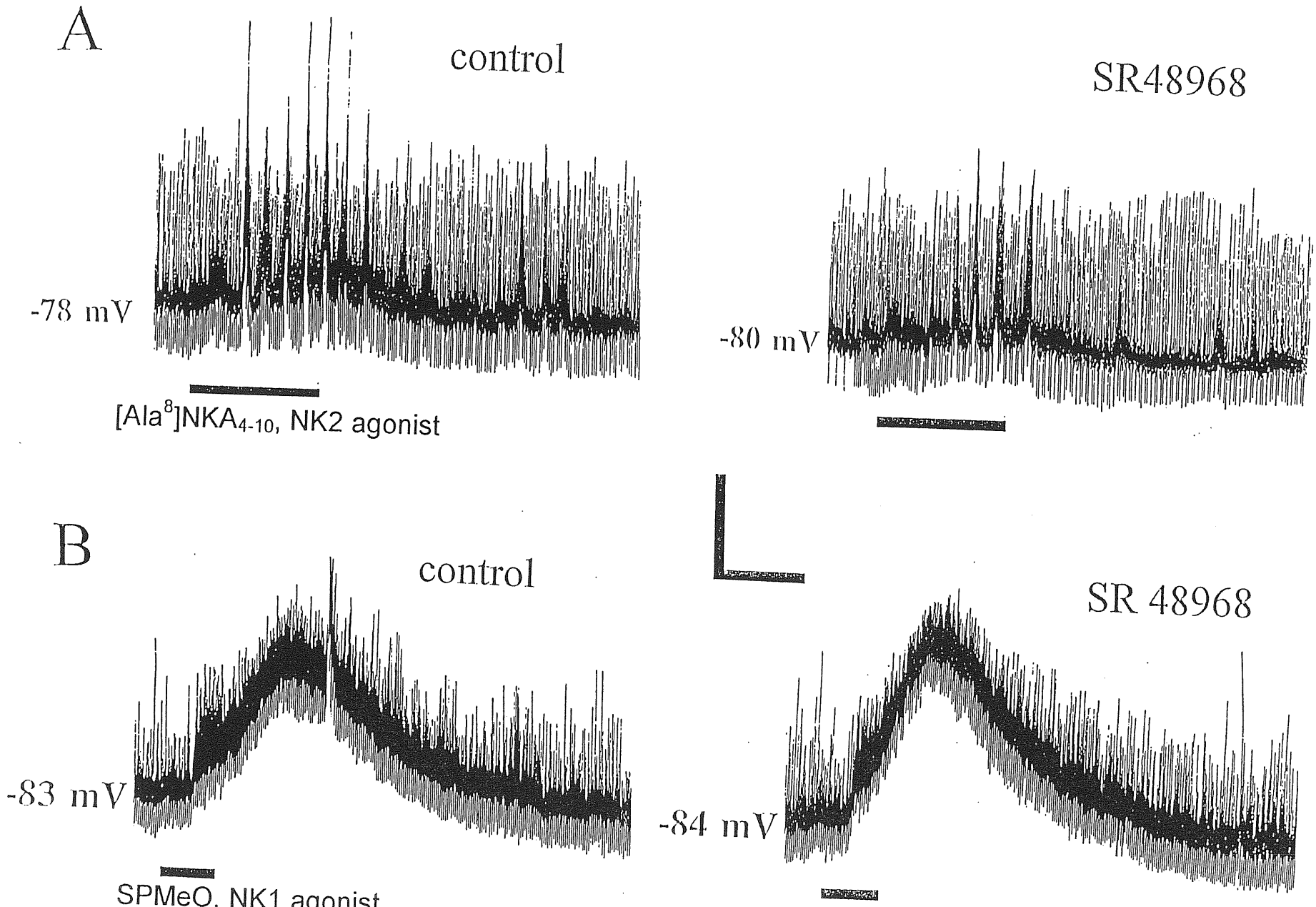
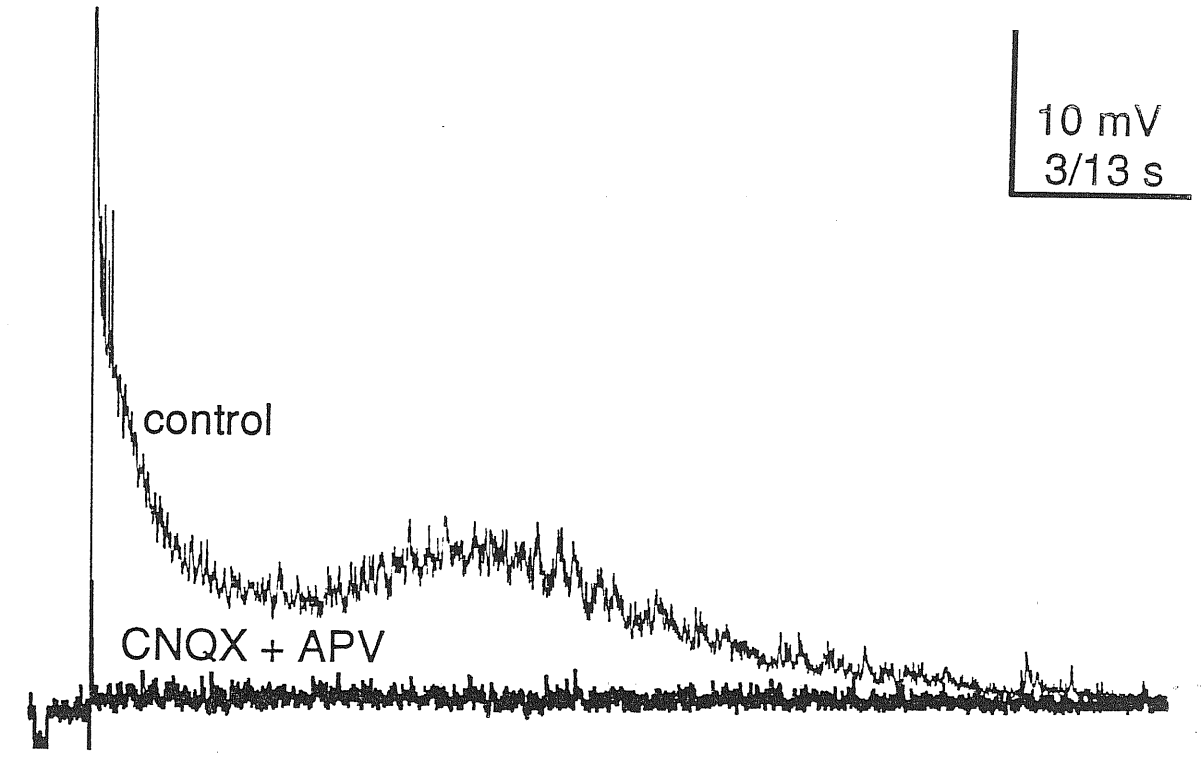

A
control
SR48968
-78 mV
-80 mV
[Ala8]NKA4-10, NK2 agonist
B
control
SR 48968
-83 mV
-84 mV
SPMeO, NK1 agonist

Fig. 13. Co-application of CNQX (10 μM) and APV (25 μM) completely blocks postsynaptic potentials evoked by dorsal root stimulation on motoneurones. The traces represent membrane potential changes in a motoneurone under current clamp. The 2 V 1 ms long single pulses in control solution evoked very long (>10 s) postsynaptic potentials (thin line in upper panel represents the average of 3 responses). After superfusion for 15 min with CNQX and APV 1ms 10 V stimulus strength failed to induce a response (thick line in the upper panel). The lower panel shows the responses in the same cell to train at 1 Hz (31 pulses) of 2 V 1 ms stimuli in control solution (thin line) and the response to train at 1 Hz of 10 V 1 ms stimuli after co-application of CNQX and APV (thick line). Note that no clear summation of postsynaptic potentials occurred in the second case. Calibration bar for time is 3 s for upper panel and 13 s for lower panel. Resting membrane potential was -81 mV.


10 mV
3/13 s
control
CNQX + APV

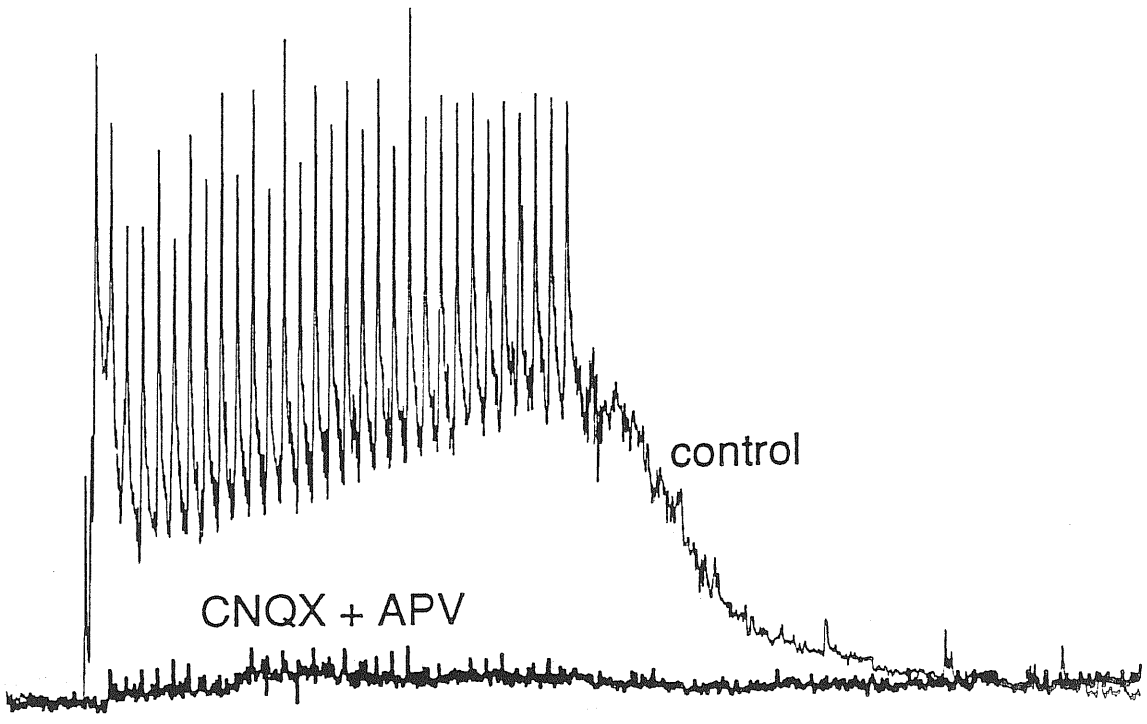
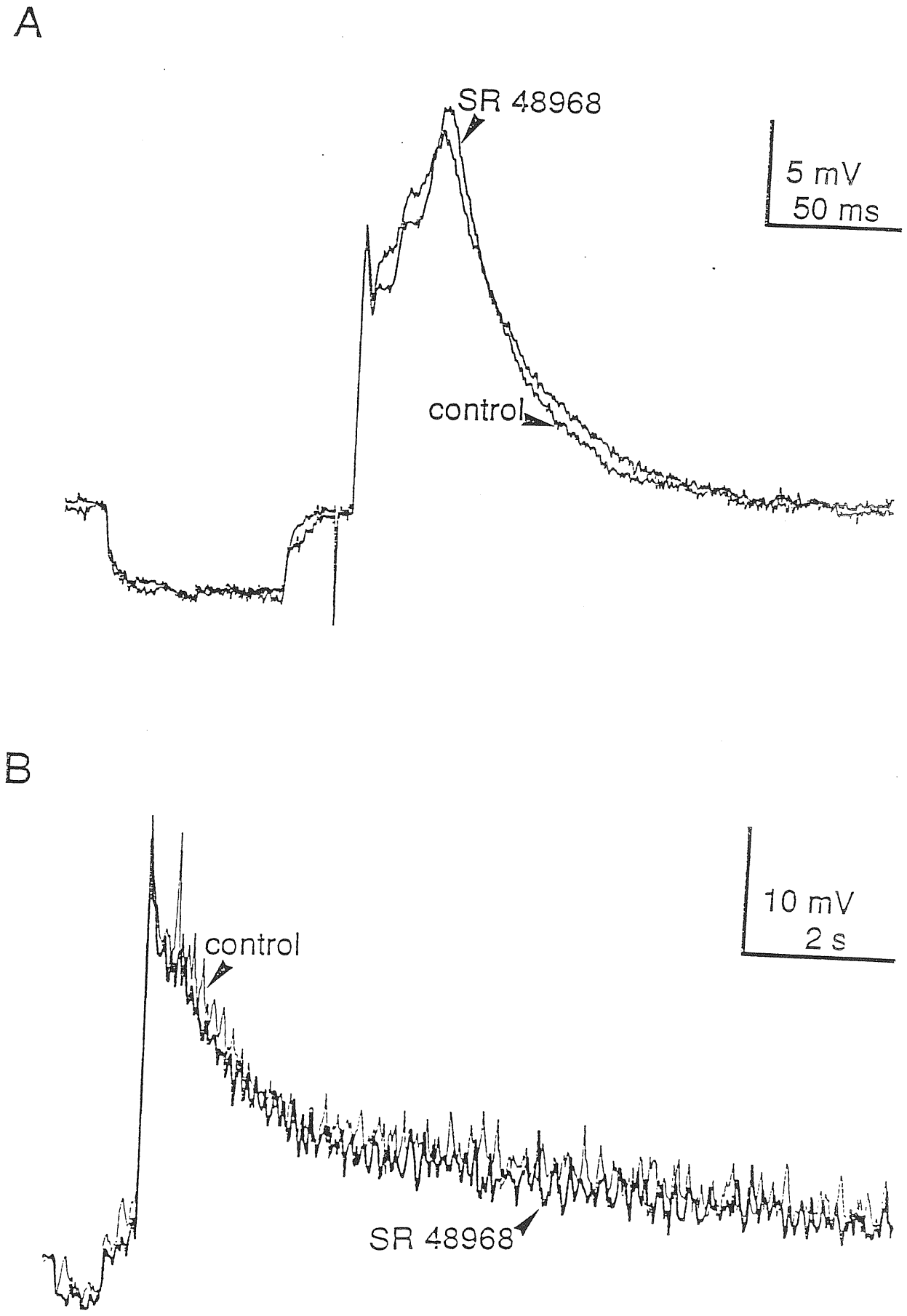

control
CNQX + APV

Fig. 14. Synaptic potentials were insensitive to the presence of SR 48968 (0.5 μM). Tracings are from the same cell. (A) Average (n = 8) of fast postsynanptic potentials (comprising monosynaptic and polysynaptic components) in control solution and after 20 min of superfusion with SR 48968 solution. The stimulus was 0.1 ms long and 2.5 V amplitude, the resting membrane potential -77 mV. The stimulus was preceded by electrotonic potential elicited by 80 ms long -0.2 nA current pulse. Note close overlap of the traces. (B) Slow postsynaptic potentials (displayed at lower gain and speed) from the same cell in control solution (faint trace) and after superfusion for 20 min with SR 48968 solution (thick trace). In this case the stimulus was 1 ms long, 10 V in amplitude and it was preceded by the electrotonic potential induced by 400 ms long -0.2 nA current pulse. The traces were filtered at 100 Hz for clarity. Note close overlap of the traces.


A
SR 48968
5 mV
50 ms
control
B
10 mV
2 s
control
SR 48968

After 20 min application of SR 48968 (500 nM) there was no change in the amplitude or duration of the low and high threshold postsynaptic potentials (Fig. 14A and B). On average, in five cells exposed to SR 48968 the amplitude and the rate of rise of the low threshold postsynaptic potentials were the same as in controls (10 ± 7 and 3 ± 5%, respectively). A similar lack of change was also found for the amplitude, area and 90-10% decay time of the high threshold postsynaptic potentials (-0.5 ± 1.5, 3 ± 4 and 0 ± 4%, respectively).

Unlike the case of SR 48968, application of SR 140333 (1 μM) did depress the low threshold postsynaptic potentials (Fig. 15A, different cell from Fig. 14). Close examination of the SR 140333 suppressive action (Fig. 15Aa) reveals that it did not change the amplitude and the rise time of fast component of low threshold postsynaptic potentials (with delay <10 ms, presumably monosynaptic) while the effect was visible only on slow component of low threshold postsynaptic potentials (with delays >10 ms, presumably polysynaptic). Pooled results demonstrated that the peak amplitude of the low threshold postsynaptic potentials determined by its slow component was reduced to 67 ± 10% ($P < 0.05$; $n = 3$).

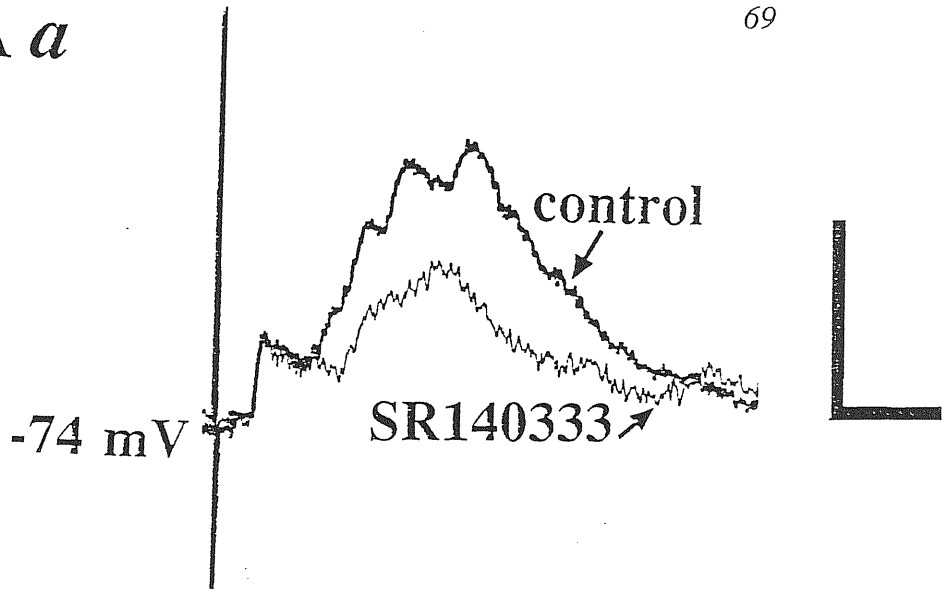
The histogram of Figure 15Ab shows that following application of SR 140333 there was also a reduction in the background *spontaneous* activity of this cell (similar data were obtained in two other cells). It suggests that there was tonic activation of NK_1 receptors which were sensitive to SR 140333. The peak amplitude of electrically-evoked high threshold postsynaptic potential was suppressed less than the one of the low threshold postsynaptic potentials (to 84%; Fig. 14B) and in pooled data from 15 cells no significant change in the peak of the high threshold postsynaptic potentials was found (95 ± 5% of the control response). Nevertheless, the decay of the high threshold slow component in the majority of cells was faster: in 10/15 cells the decay time (from 95 % to 5 % of the peak value) and the area of high threshold postsynaptic potentials were significantly suppressed (67 ± 8 and 72 ±

5% of their controls, respectively; $P < 0.05$). The effect developed during the first ~20 min of SR 140333 application - a time interval essentially the same as that needed to observe antagonism of SPMeO-mediated depolarisations. In the remaining five neurons there was no significant change (<10%) in the area and decay times of slow postsynaptic potentials even with more sustained applications of SR 140333.

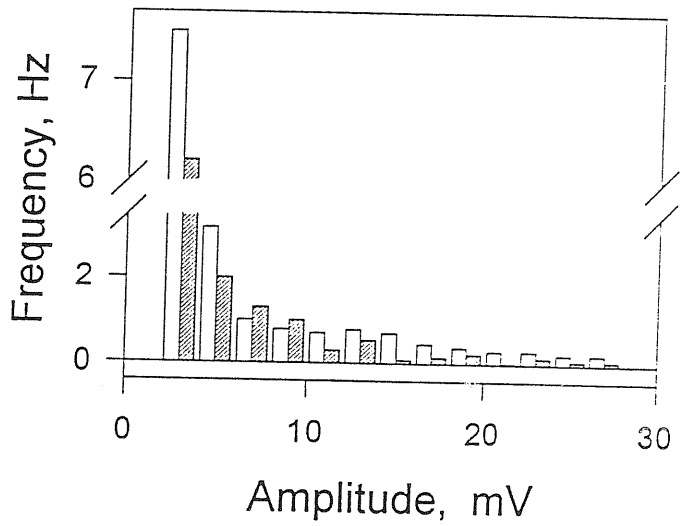
It has been shown that substance P and tachykinins can modulate glutamate responses, especially the ones mediated by NMDA receptors, and can induce glutamate release in the spinal cord (see introduction). It was therefore important to know whether the SR 140333 effect persisted in the presence of glutamate receptor antagonists, particularly in the presence of those against NMDA receptors which are believed to be involved in pain transmission (Woolf and Thompson, 1991). In the presence of the NMDA receptor antagonist APV (25 μM) the peak amplitude of high threshold postsynaptic potentials was not significantly altered (to 92 ± 5%; $P > 0.1$, $n = 4$) while there was a dramatic reduction in the area of the slow component of this response (Fig. 16A, to 24 ± 10%, $n = 4$) in agreement with previous studies (Thompson et al, 1990). Subsequent application of SR 140333 (1 μM) evoked further depression of the slow component of this response to 71% of the APV treated response (depicted at low gain in Fig. 16A) as shown in detail in Figure 16B, where the same postsynaptic potentials are displayed at higher gain. On this cell the action of SR 140333 was reversed after 30 min of washout in APV solution to 78% of the initial area of the response (not shown). In five cells, APV *per se* reduced the high threhold postsynaptic potentials area to 33 ± 5% of control. In APV solution, SR 140333 was still able to reduce the total area of the high threshold postsynaptic potentials down to 21 ± 5% of the original response in the control medium.

Fig. 15. Synaptic potentials in the presence of SR 140333 (1 μM). Tracings are from the same cell. For details of stimuli and averaging see Methods. (Aa) Fast postsynaptic potentials comprising early (presumably monosynaptic) component plus large and slower polysynaptic responses. The latter were reduced by SR 140333; to aid comparison control (thick line) and the response in the presence of drug (faint line) are superimposed. Resting membrane potential is indicated before the tracing. (Ab) Plot of spontaneous postsynaptic potential amplitude (abscissa) versus their frequency (ordinate) in control solution (open bars) and in the presence of SR 140333 (hatched bars). Note reduction in the frequency of spontaneous synaptic potentials, particularly of those of moderately low amplitude (~4 mV) and of large amplitude (>10 mV). (B) Slow postsynaptic potentials (displayed at much lower speed than in A) obtained before, during and after ~45 min washout. Fast components during the decay phase are curtailed by electronic filtering. Calibrations: A, 3 mV, 20 ms; B, 20 mV, 250 ms.

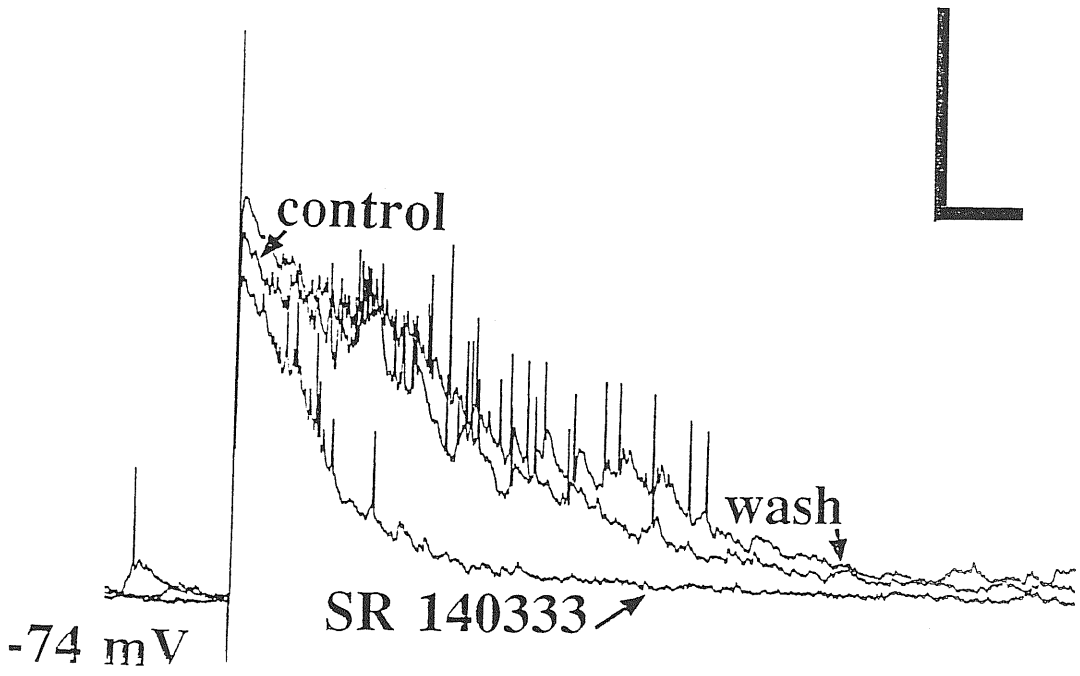
A *a*


control
SR140333
-74 mV

A *b*


Frequency, Hz
7
6
2
0
0
10
20
30
Amplitude, mV

B


control
wash
SR 140333
-74 mV

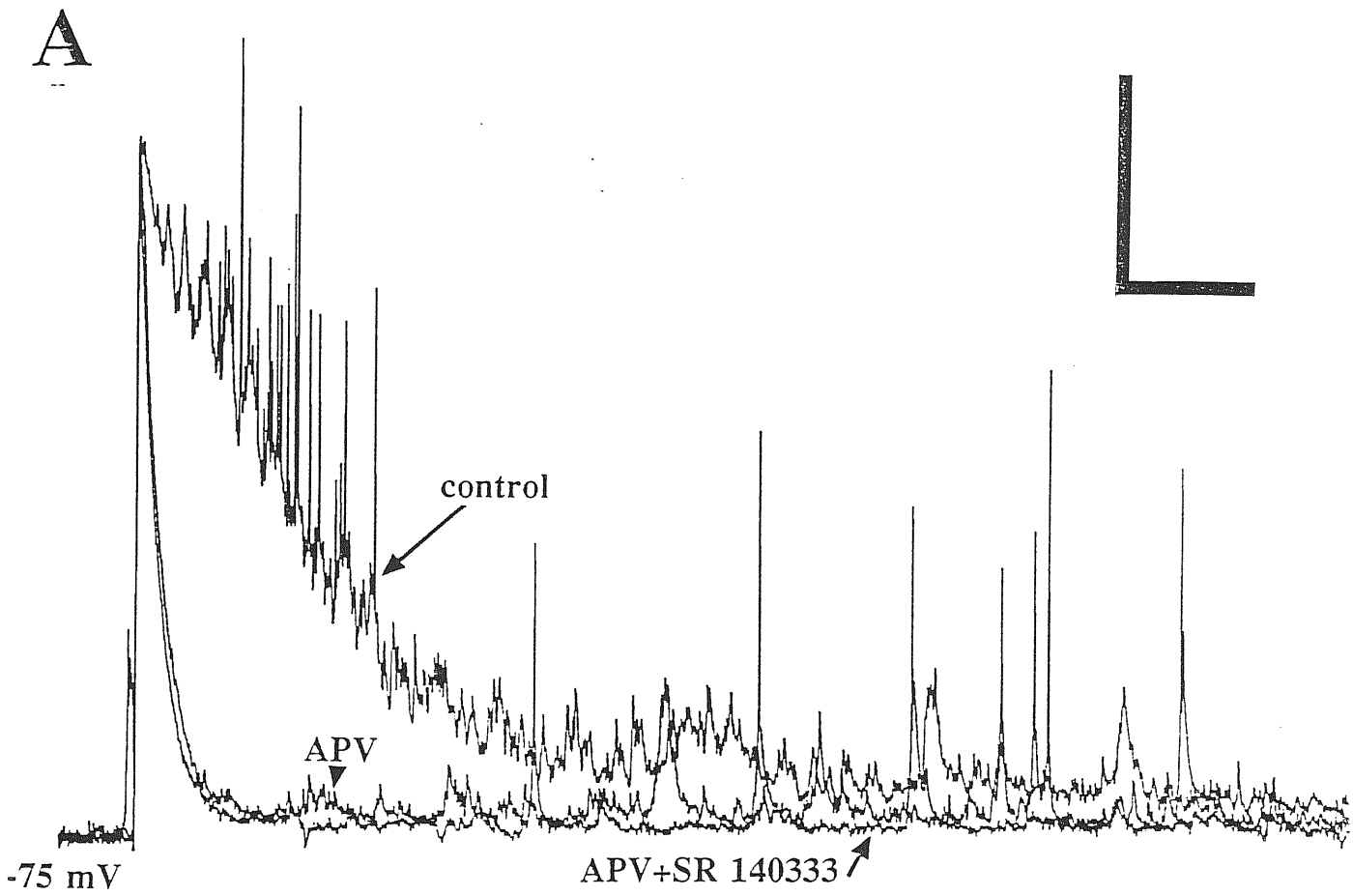
If these data are calculated taking as 100% the area of postsynaptic potentials in APV solution, the action of SR 140333 consists of a reduction to 64 ± 12% ($n = 5$; $P < 0.01$), which is approximately similar to the antagonism observed with SR 140333 alone (see previous page). On washout of SR 140333 the postsynaptic potentials gradually recovered (over ~40 min) to the level previously observed in APV solution. In practice these observations suggest a comparable fractional decrease in the high threshold postsynaptic potentials produced by SR 140333 regardless of NMDA receptor blockade. A further way to address the issue of possible interactions between glutamatergic and peptidergic systems would be to examine if the ability of APV to block the high threshold postsynaptic potentials is influenced by prior application of SR 140333: Figure 16C shows an example, in SR 140333 (1 μM) solution, of the high threshold postsynaptic potential (the area of which was 80% of the control) being further reduced by addition of APV (25 μM) to 29% of the amplitude observed in SR 140333 solution. In three cells bathed in SR 140333 solution APV decreased the high threshold postsynaptic potential area to 38 ± 9% of that before APV application. It was difficult to study the interaction between SR 140333 and a non-NMDA receptor blocker because the latter agent *per se* usually produced very strong depression (>90 %) of high threshold postsynaptic potentials. In two cells tested the area of residual response (after 10 μM CNQX application) was further reduced to 79 ± 9% by SR 140333 (see example in Fig. 17).

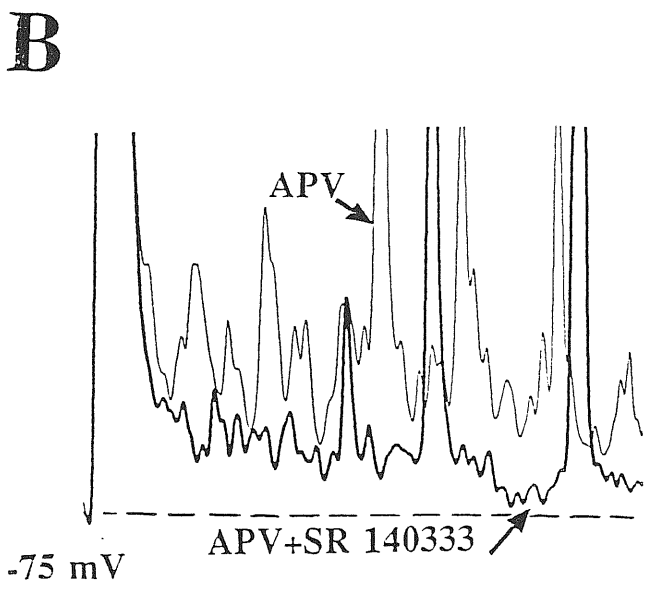
The independence of the action of tachykinins from that one of glutamate (especially NMDA) receptors is further revealed by the divergence of the effects of glutamate antagonists and tachykinin antagonists on the cumulative depolarisation underlying windup responses.

Cumulative depolarisation underlying windup responses

Action potential windup (an increase in number of action potentials after each successive stimulus of constant strength at 0.5-2 Hz frequency) is an event related to nociception and is proposed to be dependent on tachykinin and NMDA receptors (Thompson et al, 1990). It can be induced by Aδ and C fiber stimulation and is associated with cumulative depolarisation- i.e. the gradual rise in membrane potential before each stimulus reflecting a complex summation of high threshold postsynaptic potentials (see Fig. 1). In agreement with previous studies (Thompson et al. 1990, Sivilotti et al, 1993) the cumulative depolarization observed in the present study was accompanied by action potential windup in a fraction of tested cells only (13/30, 43 % incidence). In such cells a single stimulus of the same strength (duration was always 1 ms) induced a high threshold postsynaptic potential lasting >4 s, indicating that small diameter fibers were activated by this pulse. It has been demonstrated that a high rate of rise of cumulative depolarisation is correlated with the presence of action potential windup (Sivilotti et al, 1993). This was found to be the case also in the present experiments: while the rate of rise of cumulative depolarisation ranged from 0.05 to 1.18 mV/s (on average 0.36 ± 0.04 mV/s), it was significantly ($P < 0.05$; Wilcoxon test) higher (0.51 ± 0.07 mV/s) in the 13 cells having action potential windup than in those without it (0.21 ± 0.04 mV/s). Contrary to the previous findings (Thompson et al, 1990) the application of APV or CPP (NMDA receptor antagonists) did not prevent the appearance of action potential windup in 3/4 cells with action potential windup in control solution even though the underlying cumulative depolarisation was strongly suppressed (the peak decreased to 60 ± 8% of control). The positive correlation between rate of rise and action potential windup was valid also in the presence of NMDA receptor antagonists (the rate of rise was 0.54 ± 0.04

Fig. 16. Changes in high threshold postsynaptic potentials following the application of SR 140333 (1 μM) or APV (25 μM) alone or in combination. Membrane potential at rest is shown beside each tracing. (A) Control postsynaptic potential is strongly depressed by APV, which largely reduces its slow component without affecting the fast one. Application of SR 140333 in the presence of APV further reduces the slow, shallow tail of the postsynaptic potential. This phenomenon is more clearly observed in B, where gain and filtering are increased. (C) Application of APV to a cell in which the postsynaptic potential has been reduced by SR 140333 elicits a further decrease in the duration of this response while sparing its peak. A and B are from the same cell. Calibrations: A, 10 mV, 500 ms; B, 2 mV, 1 s; C, 20 mV, 2 s.


A
control
APV
-75 mV
APV+SR 140333


B
APV
APV+SR 140333
-75 mV

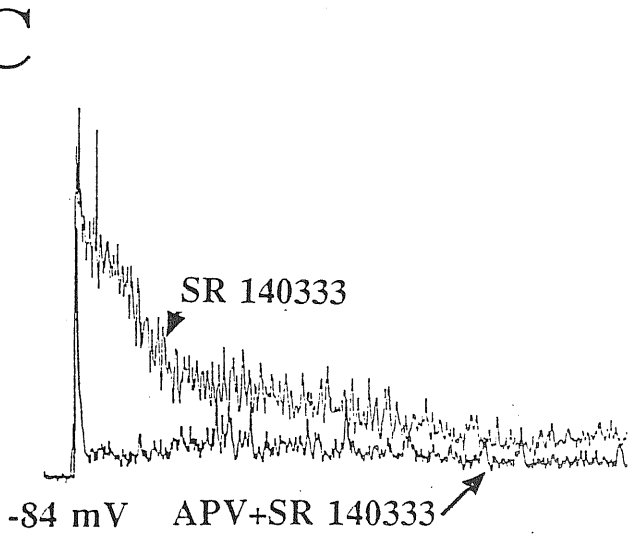
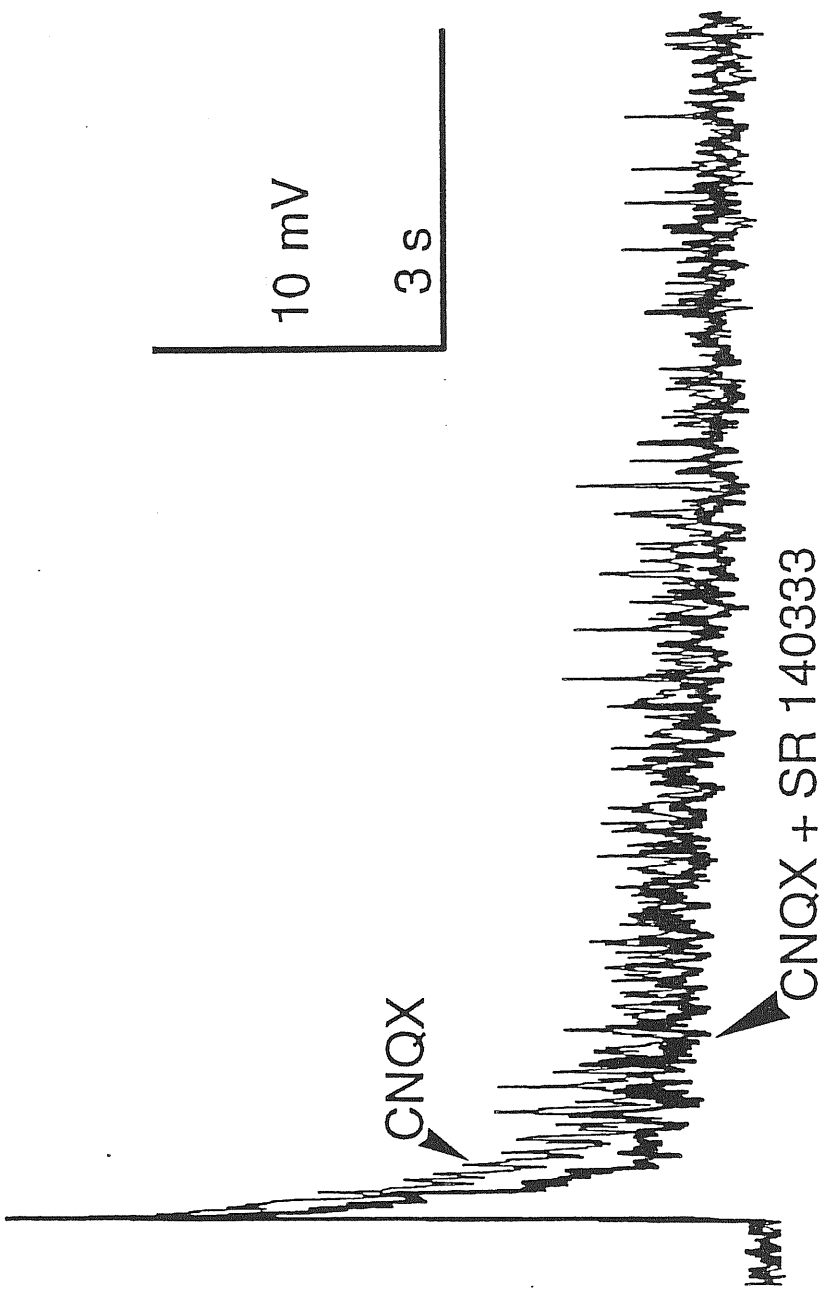

C
SR 140333
-84 mV
APV+SR 140333

Fig. 17. High threshold synaptic potentials are still sensitive to SR 140333 after application of CNQX. One ms 10 V stimulus induced, in the presence of 10 μM CNQX long and shallow postsynaptic potential (thin line), the area of which was only 15 % of the area of the response in the control solution. The subsequent application of SR 140333 (1 μM) reduced the slow tail of the response (thick line).


10 mV
3 s
CNQX
CNQX + SR 140333

mV/s in 3 cells with action potential windup while in 3 cells in which it was not observed the rate of rise was 0.20 ± 0.06). The results of antagonists on cumulative depolarisation are summarised in Fig. 18. Panel A shows in the same cell responses induced by 1 Hz train in control solution and after application of tachykinin antagonists. For clarity the traces were filtered at 10 Hz, thus the cumulative depolarisation is displayed without spikes. SR 48986 (500 nM) was applied first and then it was washed out for 40 min. This NK2 antagonist did not have any significant effect on cumulative depolarisation, a finding confirmed on four cells in which it did not change either peak (-5 ± 10 %) or the area under the decay phase (0 ± 10%) of the response. Conversely, application of SR 140333 (1 μM) did not alter the upstroke of cumulative depolarisation but attenuated the rate of rise and speeded up its decay. In all cells tested (n = 14, including those in which no effect on slow PSP could be detected) SR 140333 significantly ($P < 0.05$) reduced the decay area of such responses (to 61 ± 9% of control, $P < 0.05$) and the peak amplitude of the cumulative depolarisation (to 71 ± 8% of control, $P < 0.05$) while rapid upstroke was unchanged (to 95 ± 4% of control response). The reduction in peak was mainly due to reduction in the rate of rise (to 67 ± 7% of control response). As a high rate of rise is correlated with action potential windup, a reduction in rate of rise should result into lower incidence of action potential windup. This was indeed the case for SR 140333: in control solution 6/14 cells (43% incidence) showed action potential windup while in the presence of SR 140333 only 3/14 cells (21% incidence) possessed windup (in two cells the windup of action potentials reappeared upon washout of SR 140333). Panel B of Fig. 18 shows that APV dramatically reduced both peak and rapid upstroke of cumulative depolarisation. The decay area was also strongly reduced. Nevertheless, the rate of rise of cumulative depolarisation appeared to be even increased. On average APV (25 μM) or CPP (10 μM) slightly increased rate of rise of cumulative

depolarisation by 20 ± 7% (n = 5; P< 0.01) while all other parameters of the cumulative depolarisation were strongly reduced: rapid upstroke was reduced to 21 ± 6%, the peak was reduced to 60 ± 8%, the decay area to 63 ±. 11% (in all cases n = 5). Panel C demonstrates that the action of SR 140333 persisted in the presence of the NMDA receptor antagonist APV. On average in four cells in APV solution SR 140333 produced a reduction in the peak to 65 ± 12%, the decay area was reduced to 78 ± 10%, the upstroke to 79 ± 5% and the rate of rise was 77 ± 9% of the response in APV solution. It is clear that the action of SR 140333 on cumulative depolarisation parameters (except the rapid upstroke) was independent from the presence of the NMDA receptor antagonist. For comparison, the non-NMDA receptor antagonist CNQX strongly reduced all parameters of cumulative depolarisation (Fig. 19A, on average the upstroke was reduced to 20%, the rate of rise to 62%, the decay to 44%, the peak to 33%, n = 2), and still in these conditions SR 140333 was able to reduce the rate of rise of cumulative depolarisation (to 85 ± 5%, n = 2) while sparing the rapid upstroke as it is shown in Fig. 19B. In summary then, SR 140333 specifically reduced the rate of rise of cumulative depolarisation: since the peak of cumulative depolarisation is the sum of rapid upstroke and the integral of rate of rise during the train, the reduction in peak was only due to reduction in rate of rise while the rapid upstroke was unchanged. NMDA receptor antagonism was specific against rapid upstroke while CNQX, an non NMDA glutamate receptor antagonist reduced all parameters of cumulative depolarisation. The specificity of antagonism for different parameters of cumulative depolarisation could mean that there are several independent components of the depolarisation. Such scheme of several components is not applicable for the decay of cumulative depolarisation since it was sensitive to all antagonists tested and the effects were not additive (this topic is addressed more in the discussion.

Fig. 18. Cumulative depolarisations induced by 1 Hz stimulus are sensitive to SR 140333 (1 μM) and APV (25 μM). For details about stimuli and averaging see Methods and text. Resting membrane potentials are shown before traces. (A) Control response is unmodified by SR 48968 (0.5 μM) but is reduced by SR 140333, particularly its decay phase (all traces in A are from the same cell). To aid comparison, traces are superimposed and filtered at 5 Hz. (B) Reduction of cumulative depolarisation by APV. (C) In the same cell as in B the APVinsensitive response (amplifier gain now is doubled with respect to B) is reduced by addition of SR 140333. The responses in B and C are filtered at 100 Hz. Calibrations: 20 mV, 10 s in A and B; 10 mV and 10 s in C.

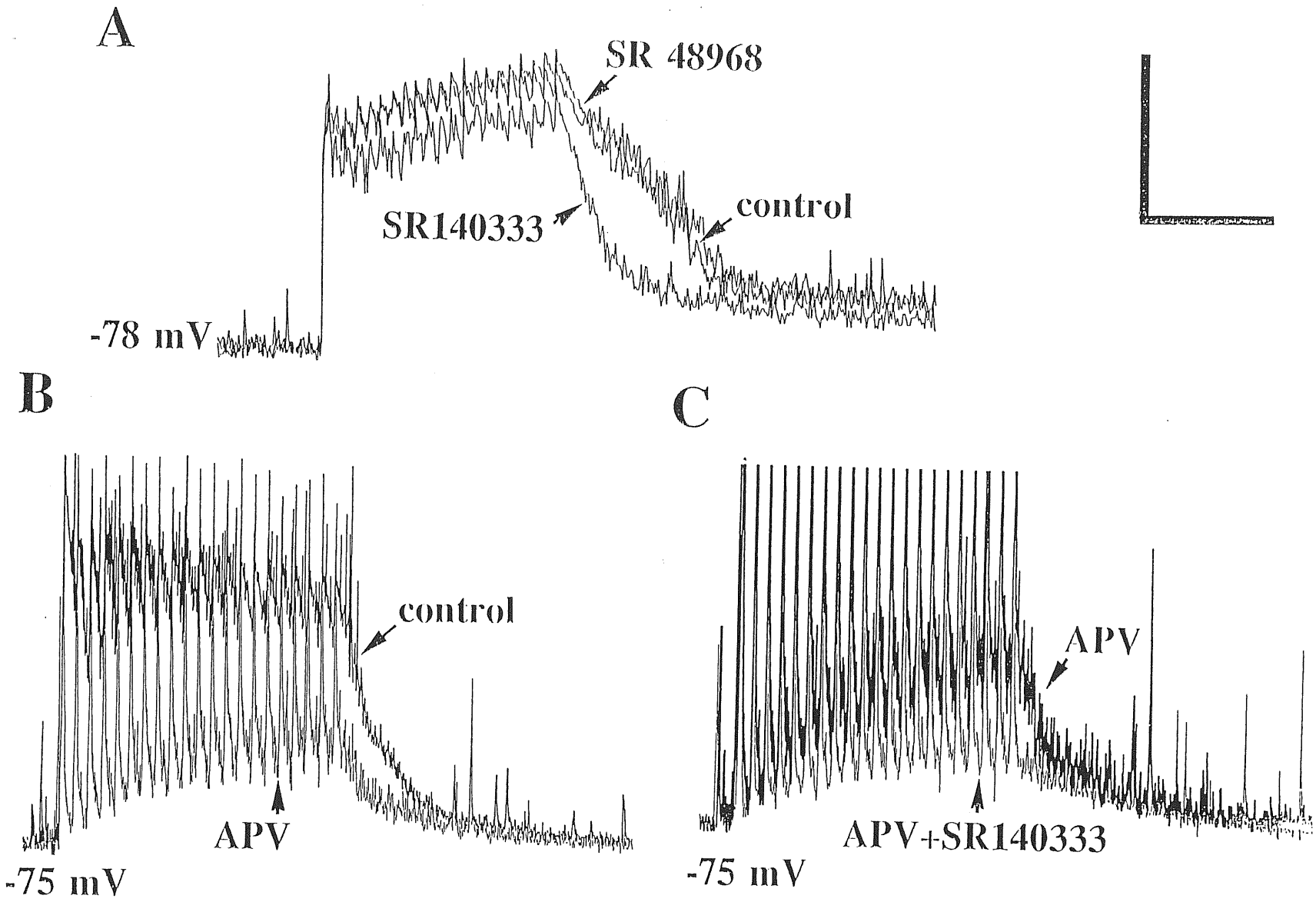
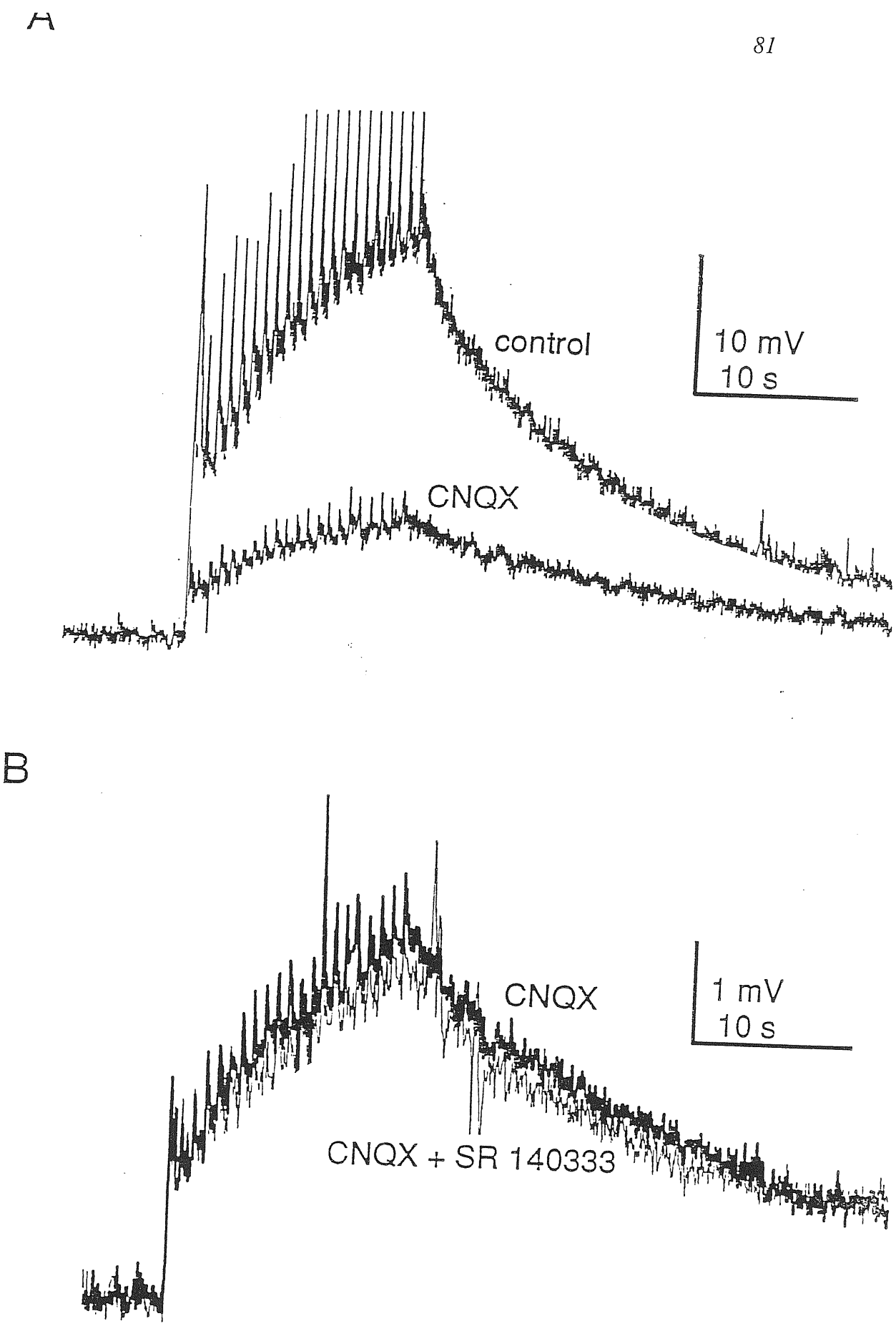

A
SR 48968
SR140333
control
-78 mV
B
control
APV
-75 mV
C
APV
APV+SR140333
-75 mV

Fig. 19. The cumulative depolarisation is still sensitive to SR 140333 in the presence of 10 μM of CNQX. (A) The train of 10 V 1 ms pulses induced rapidly developing cumulative depolarisation in control solution (upper trace). In the presence of CNQX the same train induced much smaller cumulative depolarisation (lower trace). (B) Thick line represents the lower trace from A amplified for clarity (see calibrations) and it was obtained at steady state conditions as no further reduction after 10 min continuous superfusion of CNQX was observed. The cumulative depolarisation was further reduced (thin trace) after subsequent application of SR 140333 (1 μM) for 20 min in the continuous presence of CNQX (thin trace). The reduction had all characteristics of SR 140333 antagonism: the upstroke (the initial part of the cumulative depolarisation) was not affected while the peak was reduced. Resting membrane potential was -81 mV.


A
control
10 mV
10 s
CNQX
B
CNQX
1 mV
10 s
CNQX + SR 140333

Rate of rise is known to increase with membrane depolarization and to decrease with membrane hyperpolarization in a fraction of cells (Sivilotti et al., 1993). The nature of these phenomena has not been addressed yet. Fig. 20A shows an example of the changes in parameters of cumulative depolarisation due to variation in membrane potential. The same stimulus train was applied at three different membrane potentials (-100 mV, -70mV, -57 mV) to the same cell. The parameters of cumulative depolarisation are indicated by arrows in Fig. 20A. In the same panel letter a corresponds to the upstroke, b- to the peak, c- to the area of afterdepolarisation (decay), d- to the rate of rise. The same lettering is used for graphs in Fig. 20Ba-d, where the measured values are plotted against the membrane potential. The shape of the response was changed by membrane potential changes due to differential sensitivity of various parameters: while the upstroke decreased as the cell was depolarised (Fig. 20Ba), at the same time the rate of rise and the area of afterdepolarisation increased (Fig. 20Bc and d). The peak of the cumulative depolarisation had weak voltage dependence (graph Fig. 20Bb). The behaviour of upstroke during membrane potential changes is similar to the one of the low threshold (Yoshimura and Jessel, 1990) and the high threshold postsynaptic potentials of motoneurones (as exemplified in Fig. 20C, in which the extrapolated reversal potential for the area of slow postsynaptic potential was -20 mV). This is not surprising as the upstroke is in fact the amplitude of the first response to the dorsal root stimulation 1 s after the stimulus. Conversely, the voltage dependence of rate of rise and afterdepolarisation cannot be explained by simple summation of slow postsynaptic potentials as they possess opposite voltage dependence. One of the explanations for such a voltage sensitivity of the rate of rise is the relief of NMDA receptor channel from magnesium block (Silvilotti et al, 1993). Nevertheless, the same voltage sensitivity of the rate of rise persisted in the presence of the NMDA receptor antagonists CPP or APV (Fig. 21A). Application of 20 μM CPP to this cell dramatically reduced the upstroke (by 80 % at -84 mV), the area of the single high threshold

postsynaptic potential (by 65%) and the peak of cumulative depolarisation (by 35%, data not shown) suggesting the effectiveness of NMDA receptor block. The rate of rise (which was increased by 25%) retained the same unusual voltage dependence (rate of rise is equivalent to the slope of the baseline during the train and, since the upstroke was very small with CPP solution, the rate of rise was proportional to the peak of the cumulative depolarisation; Fig. 21Aa, b): it grew from 0.015 mV/s at -100 mV to 0.55 mV/s at -84 mV. Note that, despite the application of CPP, action potential windup was present at -58 mV although, because of spike filtering, only afterhyperpolarizations can be seen (downward deflections, Fig. 21Ac). Such a strong spike activity probably impaired any further increment in the rate of rise (it was actually decreased to 0.49 mV/s). In five cells tested in the presence of CPP (or APV) and in ten cells in control solution the slope of plots of rate of rises against baseline membrane potential (prior to the train) consistently had positive value (increments in rate of rise per 10 mV change in the baseline potential were 0.09 ± 0.01 mV/s with NMDA antagonists and 0.07 ± 0.02 mV/s in control medium, respectively; data not statistically different, Wilcoxon test). On the other hand, the peak of the postsynaptic potentials decreased with membrane potential depolarisation as shown in Fig. 21B by displaying the initial part of the responses to the train on a faster time base. Nevertheless, the shallow tail after the first stimulus (indicated by arrows, Fig. 21Ba-c) grew together with the rate of rise as the cell was depolarised from -100 mV (Fig. 21Aa and Ba) to -84 mV (Fig. 21 Ab an Bb). These shallow depolarizing tails (measured 600-800 ms after the stimulus artefact) could also be observed to follow single high threshold postsynaptic potentials and they possessed the same unusual voltage dependence as it is indicated by superposition of the two traces at two different membrane potentials (see arrows pointing to this component in Fig. 21 C).

Fig. 20. Rate of rise possesses unusual voltage dependence. A and C: responses observed at three levels of membrane potential obtained by injection of 1.0 nA (a), -0.27 nA (b) and -1.5 nA (c) current in the same cell. Panel A shows the cumulative depolarisations to the trains of stimuli (1 Hz, 5.0 V, 1 ms, 21 pulse) while panel C shows high threshold postsynaptic potentials evoked by single stimulus (5.0 V, 1 ms) at faster speed. In Aa the scheme of the measurement of the parameters of cumulative depolarisation is presented: a stands for the upstroke, b stands for the peak, c stands for the area of afterdepolarisation (ADP), d stands for the rate of rise (RR). B: the measurements of each parameter (a- upstroke, b- peak, c- area of afterdepolarisation, c- rate of rise) at three membrane potentials (abscissa) are plotted versus membrane potential. Note that only the upstroke increases with hyperpolarisation and decreases with depolarisation (similarly to the high threshold postsynaptic potential evoked by single stimulus in panel C, compare the amplitude above the baselines indicated by broken lines: it decreases from c to a) while the area of afterdepolarisation and rate of rise increase with depolarisation of membrane potential. The peak has similar though much weaker dependence on membrane potential.

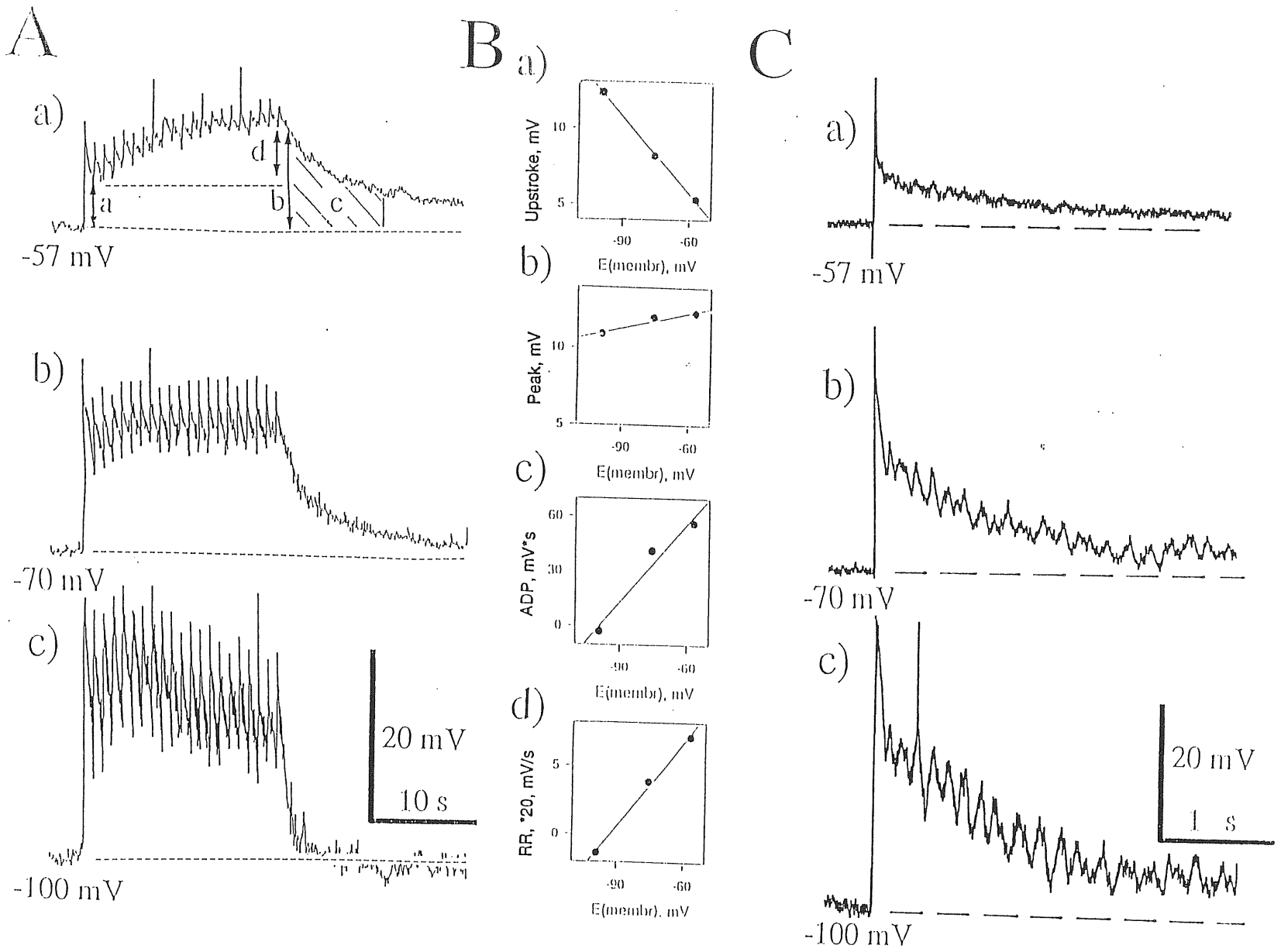
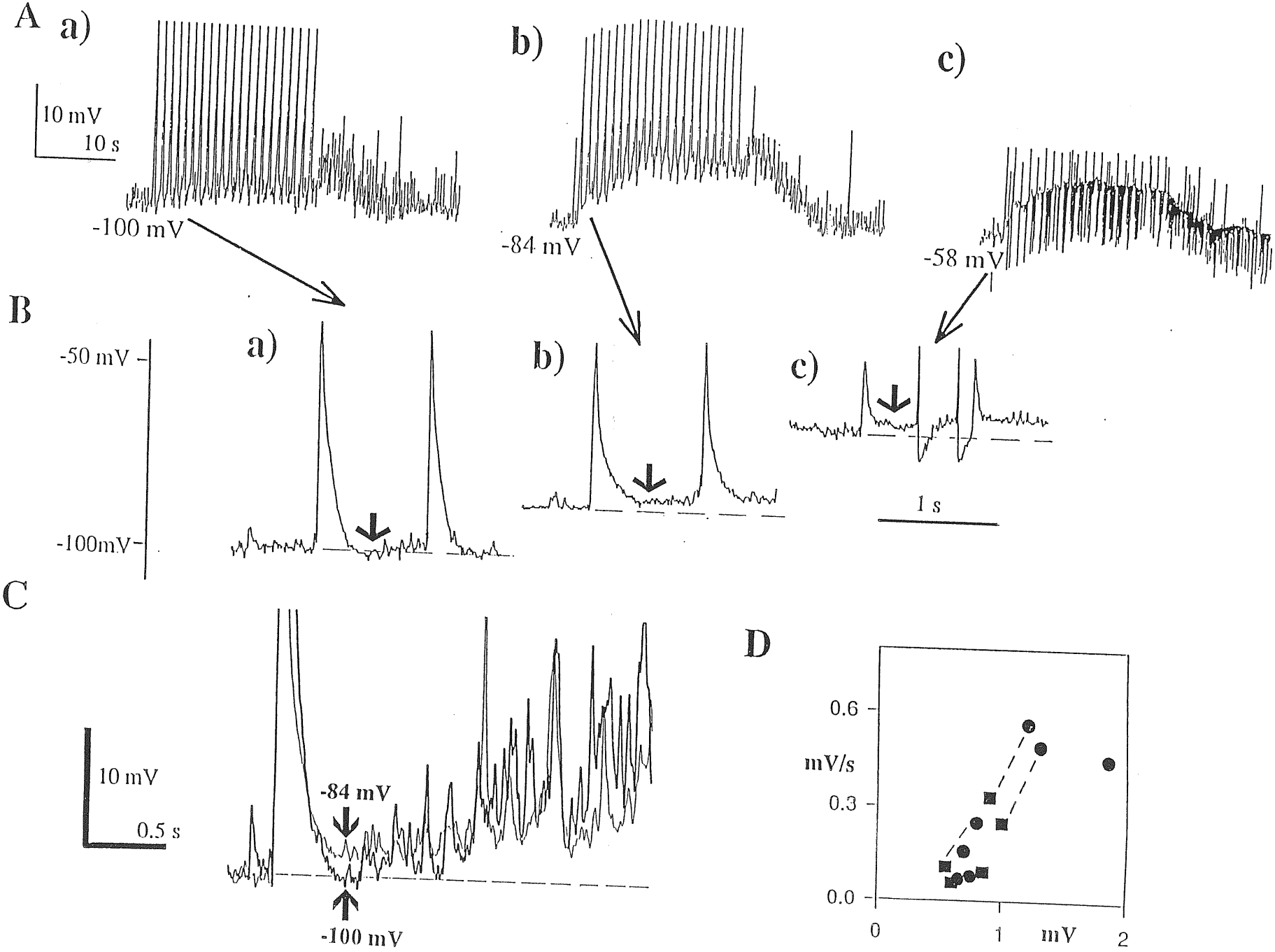

A
a)
d
a
b
c
-57 mV
b)
-70 mV
c)
20 mV
10 s
-100 mV
B
a)
Upstroke, mV
10
5
-90
-60
E(membr), mV
b)
Peak, mV
10
5
-90
-60
E(membr), mV
c)
ADP, mV*s
60
30
0
-90
-60
E(membr), mV
d)
RR, *20, mV/s
5
0
-90
-60
E(membr), mV
C
a)
-57 mV
b)
-70 mV
c)
20 mV
1 s
-100 mV

Fig. 21. Voltage dependence of rate of rise persists in the presence of the NMDA receptor antagonist CPP (20 μM). A: responses observed at three levels of membrane potential obtained by injection of -1.5 nA (a), -0.11 nA (b) and 1.0 nA (c) current in the continuous presence of CPP. Tracings were filtered at 10 Hz for clarity. In panel c) tracing contains spike afterhyperpolarizations (downward deflections). Intense spike activity (filtered out) partially obscured the true amplitude of rate of rise of cumulative depolarisation. B a-c: High threshold postsynaptic potentials recorded at the beginning of trains displayed in Aa-c. Dashed lines indicate the holding potential level. Note that at -84 and -58 mV postsynaptic potentials are followed by a slow depolarizing tail. C: high-gain record of single high threshold postsynaptic potentials evoked by the same stimulus strength used for pulse trains. Tracings of the response (from the same cell as in A,B) in the presence of CPP at two different holding potentials (thich and thin lines) are superimposed to aid comparison. Note a just visible depolarizing tail potential (arrow at -84 mV) before late activity: this component is absent at -100 mV. D: scatter plot (n = 5 cells) of rate of rise (ordinate) vs amplitude of depolarizing tail potential (abscissa). Filled circles are datapoints in the presence of CPP (or APV) while filled squares are datapoints after further addition of SR 140333 (1 μM). Dashed lines join values from the same cells at equal stimulus strength.


A
a)
10 mV
10 s
-100 mV
b)
-84 mV
c)
-58 mV
B
-50 mV
-100mV
a)
b)
c)
1 s
C
10 mV
0.5 s
-84 mV
-100 mV
D
0.6
mV/s
0.3
0.0
0
1
mV
2

In five cells bathed in CPP or APV solution this slow tail potential grew with membrane depolarization (0.7 ± 0.2 mV/10 mV membrane potential change). Fig. 21 D shows that plotting the amplitude of the slow tail potential (abscissa) versus the rate of rise (ordinate) at the same membrane potential (-79 ± 1 mV) indicated a positive, linear relation between these two parameters (r = 0.75). This relation applied to data points in CPP or APV solution (filled circles) as well as to those after subsequent application of the substance P receptor antagonist SR 140333 (filled squares). In this case the reduction in rate of rise by the NK1 antagonist was paralleled by the reduction in the amplitude of slow tails indicated by dashed lines (see Fig. 20D) which connect points from the same cell before and after SR 140333 application. These findings indicate that NK1 receptors on motoneurones could account in part for such a voltage sensitivity of rate of rise and slow tails since substance P responses had similar voltage dependence (for example, see Fig. 1) and the NK1 antagonist was specifically suppressing both rate of rise and slow tails. In order to test whether the blocking action of SR 140333 (1 μM) was in itself voltage-dependent, the rate of rise was measured in four cells at two different levels of membrane potential and the same protocol was then repeated in the presence of this receptor antagonist. Fig. 22 shows the tracings from a neurone displaying clear voltage dependence of cumulative depolarization. Rate of rise was more than two fold higher at -59 mV (top) than at -74 mV (bottom) membrane potential (0.43 mV/s and 0.18 mV/s, respectively). Nevertheless, the fraction reduced by SR 140333 changed only from 76 % at -74 mV to 67% at -59 mV. Pooling data from 4 cells shows that the action of SR 140333 was to depress the rate of rise to 56±16 % or to 64±12 % at resting (-76±3 mV) or at -60±4 mV membrane potential, respectively. The similarity of these values indicates that the fractional block by SR 140333 was rather voltage-insensitive and confirms that SR 140333 is a specific agent for the rate of rise of cumulative depolarisation.

Intrinsic voltage and time dependent conductances might also contribuite to the rate of rise and to its sensitivity to membrane potential. In this case, the fraction of active conductances generating the rate of rise should be expected to be proportional to the integral over the time of the rate constant for conductance activation. If this relation applies to the present data, as a first approximation the area (measured as voltage*time) of individual high threshold ostsynaptic potential or of the cumulative depolarization should be correlated to the rate of rise. Fig. 23 shows that, in control solution, relatively weak pulses (1 ms; 1 or 2 V, thin or thick trace, respectively) generated postsynaptic potentials lasting several s. When the same pulses were used for stimulus trains there was either a decrementing depolarization (after the first 1 V stimulus) or a modest cumulative depolarization starting after the 5th pulse in the 2 V train (Fig. 23 Ab). In the presence of APV single postsynaptic potentials evoked by the same stimulus strengths were reduced as their area were 15 and 12 %, respectively, of controls (Fig. 23 Ba) due to the large reduction in their slow component (cf. Thompson et al., 1990; Baranauskas et al, 1995). When these stimuli were applied in a train, the resulting response had the rate of rise with a much larger positive value (0.70 mV/s) for the 2 V pulse train than for the 1 V one (0.1 mV/s; see Fig. 23 Bb). Note that, even if the area of the 1 V postsynaptic potential in control solution was much larger (103 mV*s) than the 2 V one in APV solution (18 mV*s), the 1 V train in control solution produced a decrementing depolarisation (-0.2 mV/s) while the 2 V train evoked modest rate of rise. This difference could not be ascribed to a difference in the total number of spikes in the train (45 for 1 V stimuli vs 48 for 2 V stimuli; the former train had a decrementing number of spikes/pulse while the latter had spike windup) or to a difference in cumulative depolarization area (208 and 240 mV*s, respectively). In order to isolate the NMDA receptor-dependent component of the response to 2 V train, the responses to 2 V pulses in APV solution were subtracted from the corresponding ones in control solution.

Fig. 22. Effect of SR 140333 on rate of rise at two holding potentials.

Top and bottom tracings are from the same cell at two different potentials (indicated before each trace obtained following injection of 1.2 and -0.19 nA, from top to bottom, respectively) before (control; thick line) and after application of SR 140333 (thin line). Rate of rise is increased by depolarization but the fractional block by SR 140333 remains similar.

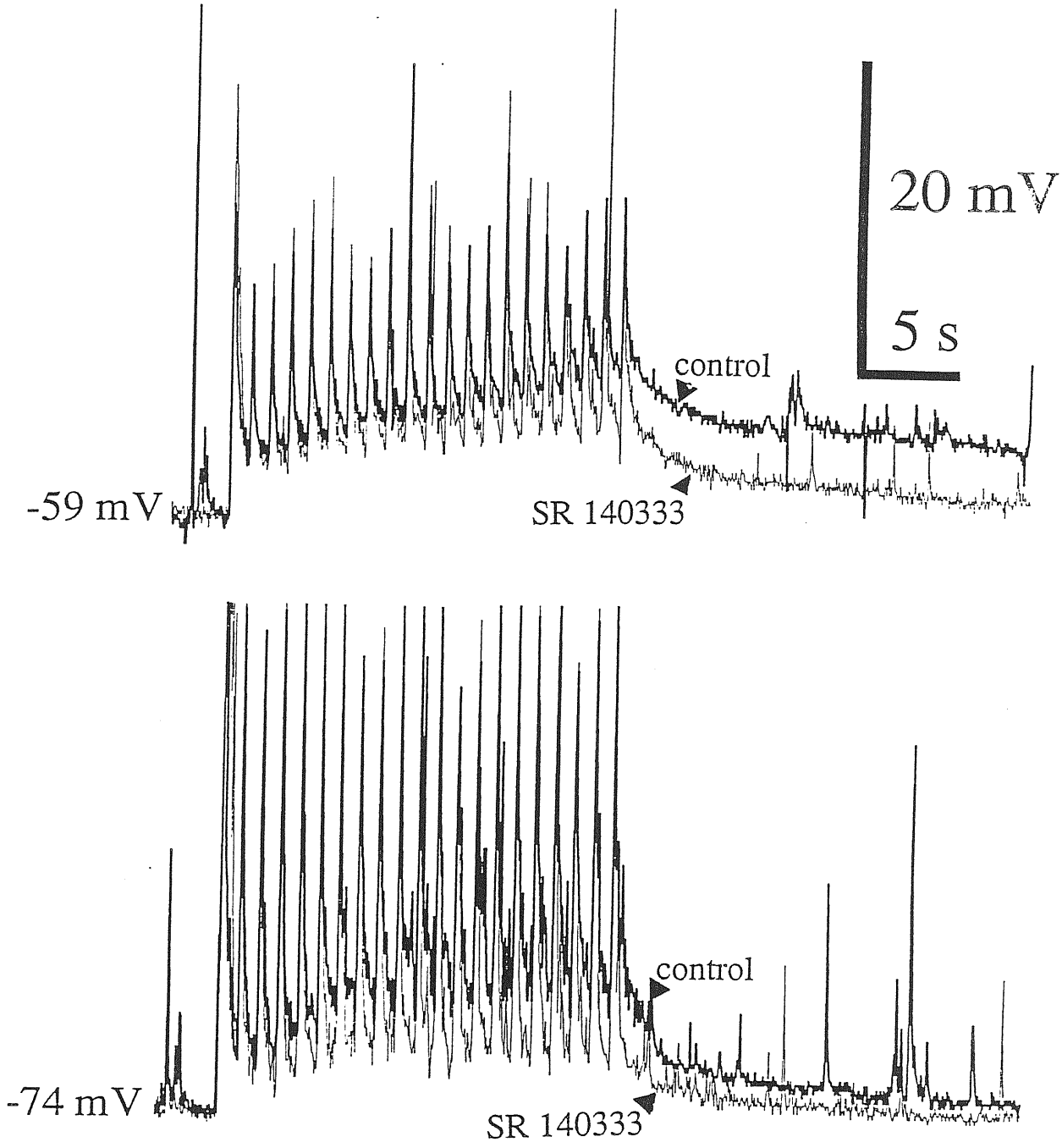

20 mV
5 s
control
-59 mV
SR 140333
control
-74 mV
SR 140333

Fig. 23. Dependence of rate of rise on stimulus intensity applied to dorsal root fibres.

Aa: superimposed single long postsynaptic potentials elicited by 1 (thin line) or 2 V (thick line) stimulus (1 ms) applied to dorsal root in control solution. Ab: responses induced by pulse train. Note that 1 V pulse is unable to evoke sustained depolarization and produces a decrementing response with negative rate of rise. Ba: superimposed single long postsynaptic potentials induced by the same pulses in the presence of APV (25 μM): note large reduction in slow component of postsynaptic potentials while peak is spared. Bb: 2 V pulse train evokes incrementing depolarization with high rate of rise (thick trace) while 1 V train has only slight effect. C: superimposed tracings of response obtained by subtracting the 2V response in APV from the 2 V one in control solution (in order to demonstrate the NMDA receptor sensitive component) and the 1 V response in control solution. These are nearly identical and are manifested as a decrementing depolarization. The vertical calibration bar for voltage applies to all tracings. All data are from the same cell at -77 mV membrane potential.

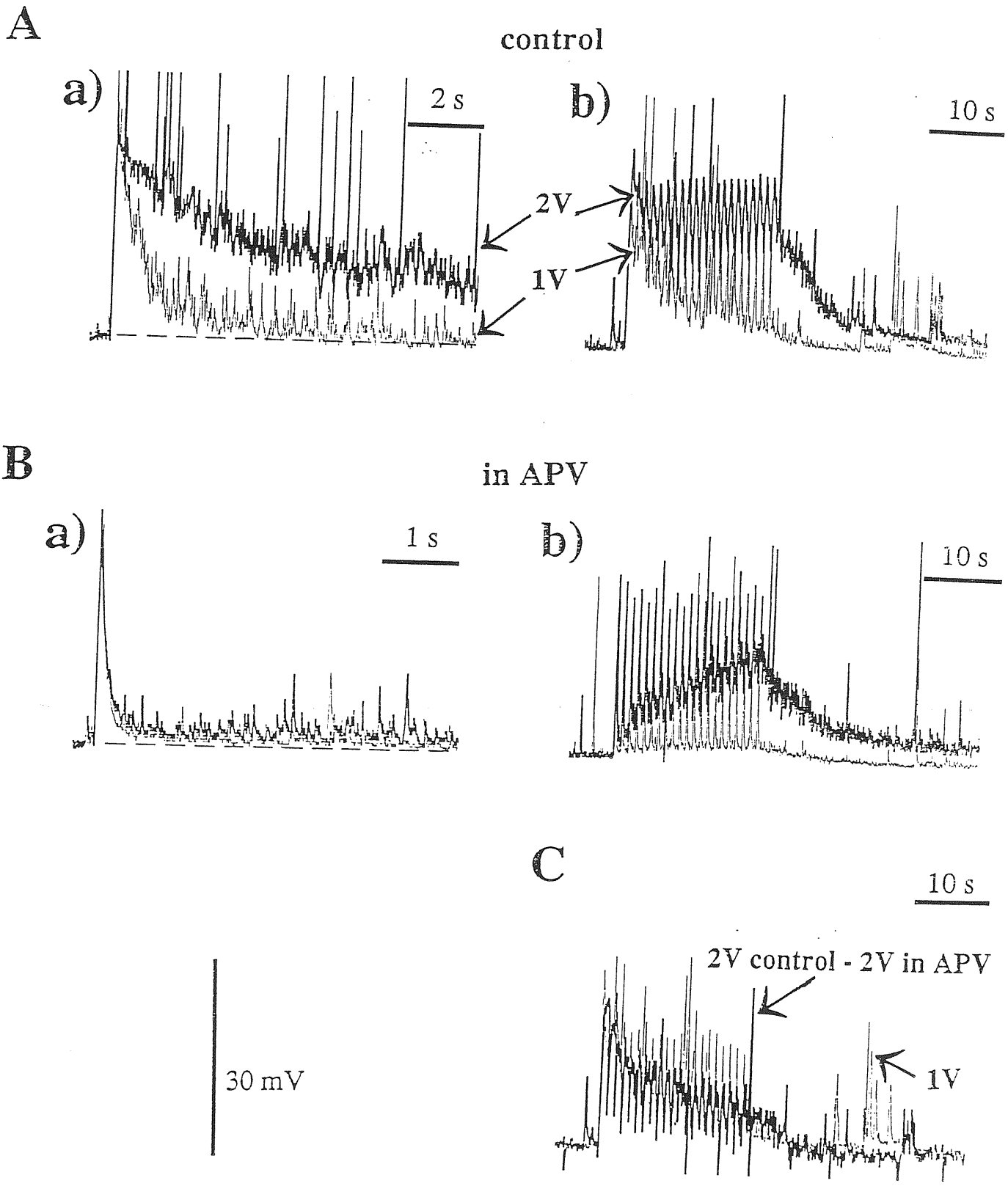
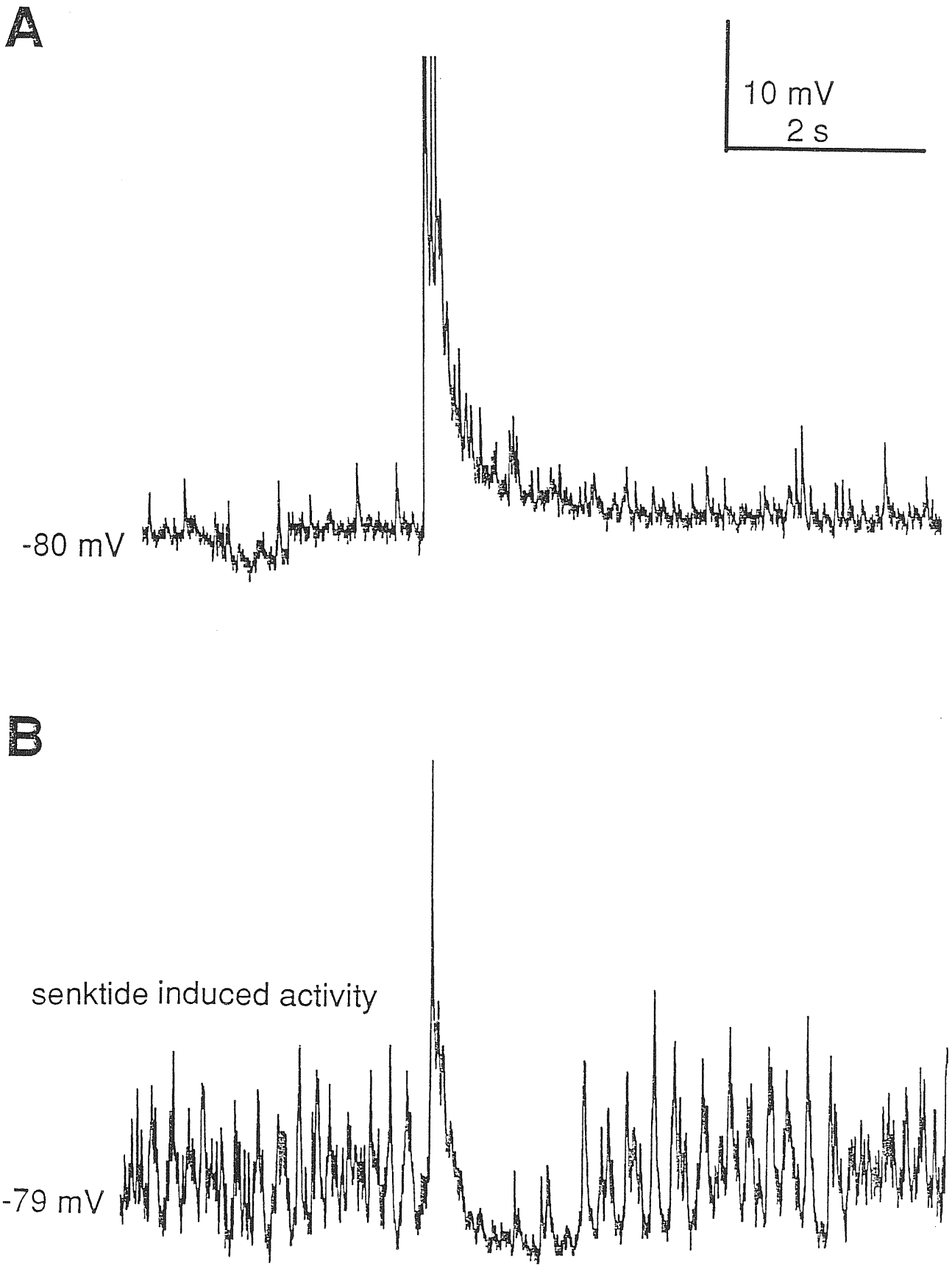

A
control
a)
2 s
b)
10 s
2V
1V
B
in APV
a)
1 s
b)
10 s
C
10 s
2V control - 2V in APV
1V
30 mV

Fig. 24. A stimulus able to induce a long high threshold postsynaptic potential (~3 s) can produce long lasting suppression of agonist evoked synaptic activity. A. In control solution 1 ms 4.0 V stimulus induced long (3 s) postsynaptic depolarising potential. B. The application of 200 nM of senktide (an agonist at NK_3 receptors) for 5 min induced sustained (> 10 min) increase in background synaptic activity (an oscillation-like behaviour indicated by the arrowhead). The same stimulus was then applied. Under conditions of intense ongoing synaptic activity stimulus induced long lasting (~1.5 s) almost complete suppression of background responses after peak of evoked postsynaptic potential (arrowhead).


A
10 mV
2 s
-80 mV
B
senktide induced activity
-79 mV

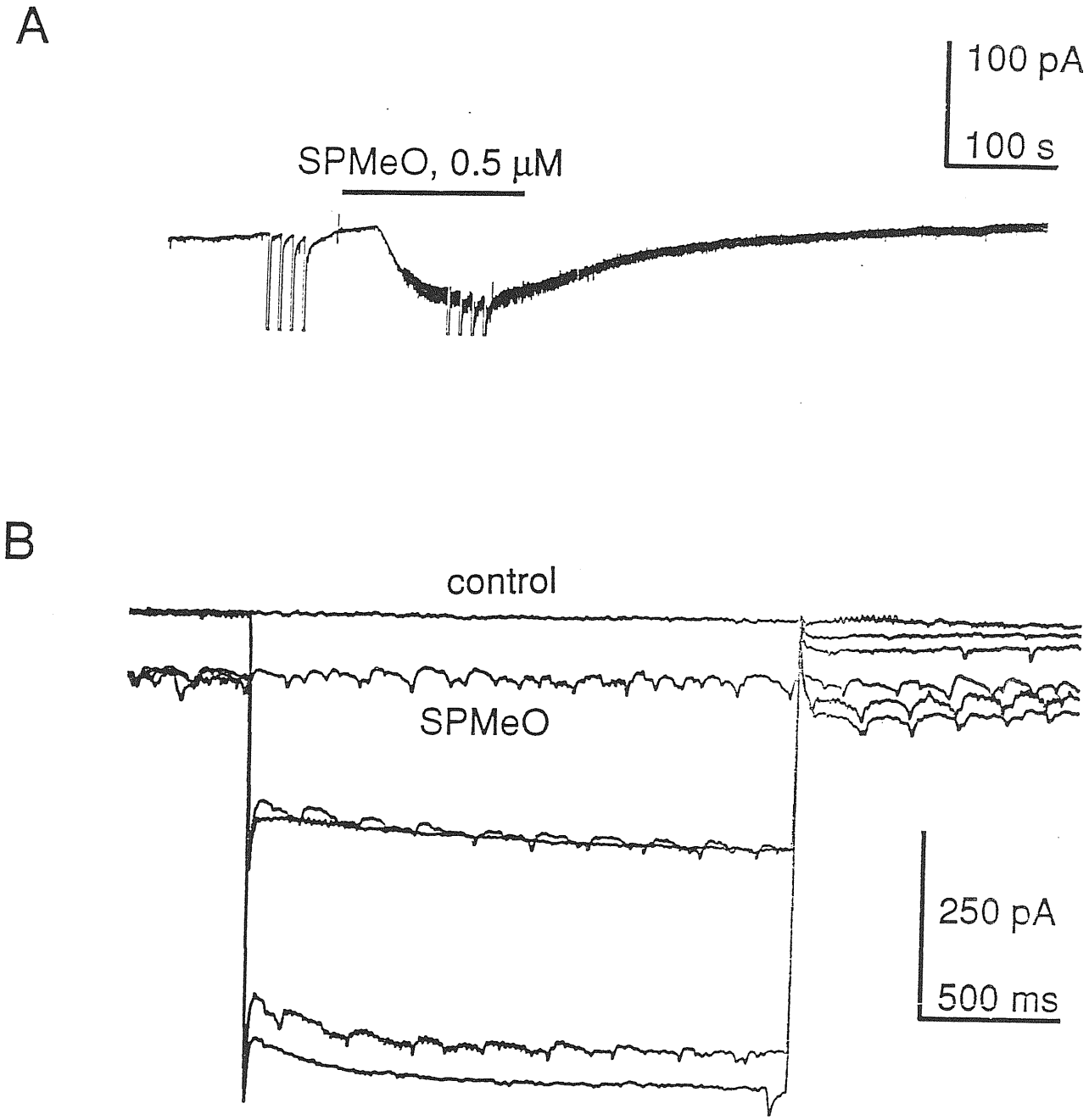
The difference is shown in Fig. 23 C and superimposed on the tracing produced by 1 V train in control solution. The slow depolarization was very similar in either case indicating that the NMDA receptor component corresponded to the slowly decrementing depolarisation in control solution. These observations were obtained from three cells which all displayed action potential windup. In summary then, the ability to express rate of rise was not merely related to the amplitude and duration of the EPSPs or cumulative depolarization but to a particular strength of afferent fibre stimulation.

The lack of any evident relationship between the parameters of single synaptic potential in control solution and the speed of their summation during the train (the rate of rise, Thompson et al, 1990, Sivilotti et al, 1993) suggest that there was a decrease in the area of summating postsynaptic potentials. Indeed, on average the area of the last postsynaptic response in the 20 s long train was only 31 ± 3% (n = 7) of the first one. It is interesting to note that, in the presence of NMDA receptor antagonists, this reduction was much less (in this case the area of the last response was 75 ± 8 % of the area of the first one, n = 6). It additionally suggests that the NMDA receptor mediated component was more prone to depression since it was reduced strongly during the train. This depression of synaptic transmission was not easily observable following single pulses under control conditions (Fig. 24A). Nevertheless, when background synaptic activity was enhanced (in this example by application of senktide), the same single pulse elicited a postsynaptic potential followed by a phase of depression of synaptic activity (Fig. 24B). Perhaps analogous depression developed during cumulative depolarisation even if the cellular mechanisms involved remain to be clarified.

Part II. Currents induced by substance P and the NK1 agonist substance P methyl ester in brainstem motoneurones

This study was done on 24 motoneurones in the hypoglossal nucleus of the brainstem. In these cells several types of calcium currents and a cationic current (slow inward rectifier) were described in previous studies (Bayliss et al, 1994). In addition, intracellular recordings indicated that various classes of outward current should also be present (Viana et al, 1993).The motoneurone input resistance was 100 ± 25 MΩ while the resting potential was -60 ± 5 mV. Substance P (2-10 μM) and substance P methyl ester (0.1-1 μM) could induce clear responses only in a small percentage of the cells (4/11 and 3/13 respectively). There was no correlation beween the presence of GTP in the patch pipette and likelihood of observing responses (3/11 responses in the presence of intracellular GTP and 4/24 in the absence), neither with preparation age or cell input resistance. In responsive cells substance P and substance P methyl ester induced a slowly developing (see Fig. 25A) inward current (from -70 mV holding potential) which started to decay during the application of the agonist and returned to the baseline after 10 min of wash out. Downward deflections correspond to membrane currents induced by voltage commands used to construct I/V curves. The thicker part of the trace represent, perhaps, not an increase in synaptic activity but dendritic spikes: their frequency was changed by hyperpolarising pulses (Fig. 25B). In the majority of the responses (3/4 for substance P and 2/3 for NK1 agonist) the current underlying the response reversed at -90 ± 5 mV and was inward at the resting potential (Fig. 25B and C). In all cases no detectable reduction in slow inward rectification was observed as it is clear from the example of Fig. 25B: two lowest traces (induced by the largest negative pulses from -70 mV to -150 mV) during the pulse display the

Fig. 25. Substance P induced an inward current in hypoglossal motoneurones. (A) The current recording trace of voltage clamped motoneurone (holding potential was -70 mV). The application of SPMeO for 3 min (indicated by horizontal bar) induced slowly developing inward current with superimposed membrane potential oscillations (seen as the thickening of the trace). The inward current peaked before the start of washout and slowly (~10 min) decayed during washout. (B) The long (2 s) negative (-110 and -150 mV) voltage pulses were used to build I-V curve of the neuron in control solution and during the response. Two upper lines represents the baseline recordings in control solution and in the presence of SPMeO (with membrane potential oscillations) and lower lines represent the current induced by voltage pulses. On the right hand of the traces the part of tail currents is seen. Note voltage activated slowly developing inward current which has the same time course in control solution (smooth trace) and in the presence of SPMeO (the trace with oscillations). Leak current was not substracted. (C) The plot of current amplitude (taken at the start of the voltage pulse immediately after capacity transients) versus the holding potential in control solution and in the presence of SPMeO. Note that graphs intersect at about -100 mV indicating the reversal potential of the current induced by SPMeO.


A
100 pA
100 s
SPMeO, 0.5 µM
B
control
SPMeO
250 pA
500 ms
C
0 pA
-70 mV
-500 pA
-250 pA
SPMeO
-95 mV
-120 mV
control
-145 mV

same time course (apart from the steady current shift) indicating that slow voltage activated conductances were unchanged. In one cell there was no clear reversal potential and in an other one the reversal was around -40 mV (the response was outward at -60 mV holding potential).

DISCUSSION

The principal findings described in the present thesis are:

first, the demonstration with highly specific agonists that all three tachykinin receptor subtypes are present and functional in the spinal cord of the neonatal rat. It is likely that each of them has a different role as the responses to each agonist had different characteristics;

second, the present experiments provide evidence that the nonpeptide antagonists developed for peripheral tissue receptors are not always effective on the central nervous system (for example, RP 67580 and CP 99994); nevertheless, two antagonists were found to be effective and quite selective (SR 140333 for NK_1 receptors and SR 46968 for NK_2 receptors);

third, from experiments with potent antagonists it was possible to demonstrate that: a) the NK_2 SR 46968 sensitive receptors play a minimal role in synaptic transmission from dorsal roots to motoneurons; b) the NK_1 receptors (blocked by SR 140333) can be tonically active and thus able to modulate fast postsynaptic potentials in addition to long ones;

fourth, NK_1 receptors seem to be especially important for slow synaptic potential summation at low (0.5-2 Hz) frequencies of dorsal root stimulation and consequently for the action potential windup; their role is apparently independent from NMDA receptors.

These results suggest an important role of substance P and other slow neurotransmitters (still to be determined) in the synaptic integration of nociceptive information in the range of seconds via summation of slow postsynaptic potentials independent from ionotropic glutamate receptor activation.

In the following sections each of these topics will be discussed in detail.

Tachykinin receptor subtypes in the spinal cord of neonatal rat

Previous autoradiographic studies (Beresford et al, 1992) showed that there are abundant NK_1 receptors in the spinal cord of the rat (both in dorsal horn and ventral horn) while the presence of NK_3 receptors is restricted to the ventral horn of neonatal rat spinal cord. The present experiments with tachykinin agonists allow to conclude in addition that NK_1 and NK_3 receptors are functional. The experiments with TTX solution (which helps to isolate the response of recorded cell from the presynaptic effects) strongly suggest that NK_1 receptors are always present on motoneurones (confirming previous data based on extracellular recordings, Ireland et al, 1992) while NK_3 receptors are present in 30% of motoneurones only (this finding could reflect an intermediate developmental state). The experiments with the NK_2 agonist strongly suggest that NK2 receptors are also present and functional in the neonatal spinal cord (mainly on interneurones as the responses to the NK_2 receptor agonist were largely suppressed by TTX). This is important in resolving the discrepancy between the data obtained from binding studies (which showed in some cases the presence of NK_2 receptor like-binding, Yashpal et al, 1990; but not in others, Watling and Krause, 1993) and in situ hybridisation (which did not find any significant amount of NK_2 mRNA, Tsuchida et al, 1990). The subsequent autoradiographic study with the NK_2 receptor antagonist SR 46968 confirmed the presence of small amount of NK_2 SR 48968 sensitive receptors (Baranauskas et al, 1995). Their low level of expression (in agreement with physiological studies) could explain negative results from in situ hybridisation experiments.

Another important aspect of the experiments with agonists was to indicate the distinct characteristics of the responses due to each subclass of receptor activity. The responses to NK_1 agonists were usually smooth, slow depolarisations while those to NK_3 agonists were regular bursts. NK_2 receptor agonist-mediated responses were intermediate and often the depolarisation was minor. Bursts were blocked by TTX and could not be induced by simple depolarisation to similar levels of membrane potential, indicating a network based mechanism for the burst generation. The presence of bursts suggest a complex modulation of the synaptic circuitry. Additional evidence for this stems from the following observation: NK_2 agonists were able to increase input resistance of motoneurones, an effect absent in TTX containing solution. Thus, NK_2 agonists (perhaps like other tachykinins) are not simply increasing the excitability of all interneurones: the prevailing transmitters in the spinal cord are glutamate and GABA and their massive release would induce the opening of ligand gated ionic channels and consequently an increase in conductivity of motoneurone (thus, they are expected to decrease the input resistance of the cell). The increase in input resistance of motoneurones indicates that other transmitters besides glutamate and GABA were released upon these cells (substance P, TRH etc; capable of inducing input resistance increase, Fisher and Nistri, 1993). Thus, a certain population of interneurones, yet to be identified and containing these transmitters (substance P, TRH etc), was presumably activated by tachykinin agonists. This view is in agreement with in vivo studies which demonstrate very specific roles for each receptor (Laneuville et al, 1988; Maggi et al, 1993): for instance, injection of NK1 receptor agonists is able to induce biting and licking of the hindlimbs of rats while NK3 receptor agonist injection increased reaction time. Moreover, the burst activity during the responses is reminiscent of the one observed in embryonic chick motoneurones (Hamburger and Balaban, 1963) and during induced fictive locomotion in rats (Kudo and Yamada, 1987; Smith and Feldman, 1987; Cazalets et al, 1992). It suggest that tachykinins

might modulate locomotion driving rhythm in the spinal cord of neonatal rat. Further studies with selective antagonists applied during fictive locomotion might help to answer this issue.

Tachykinin receptor antagonists RP 67580 and CP 99994

The negative results with these two antagonists could be due to several reasons. The simplest explanation might be that these antagonists could not reach receptors because of restricted accessibility. This seems to be an unlikely explanation as no activity of antagonists was observed even after one hour of continuous application. Most of the tachykinin agonists have similar complexity of structure, yet they can activate motoneurones within minutes. The concentrations used are several fold higher than the ones needed for receptor saturation (estimated from the binding studies, Regoli et al, 1994). Therefore, the most likely explanation for the lack of effect of these antagonists is that spinal NK_1 receptors somehow differ from peripheral ones. It is contradictory to in situ hybridization data which show the presence of the same NK_1 receptor mRNA in the spinal cord and in the periphery. Nevertheless, the existence of other types of mRNA cannot be excluded; postranslation processing could be responsible for the difference. This data suggest that any drug designed for peripheral tissue receptors should not be assumed to work on central ones as well and should be tested directly for its activity on central neurones.

Since in vivo experiments show some analgesic effects of RP 67580 (Watling, 1992) the experimental demonstration of the lack of its effect on synaptic transmission was even more surprising. A possible explanation could be that these effects were actually exerted in peripheral tissues or/and some nonspecific action could be responsible for that. Another possibility is suggested by Thompson et al, 1994: tachykinin receptors sensitive to RP 67580 and CP 96345 (which has similar chemical structure to CP 99994) are involved in synaptic

transmission from dorsal root fibers to motoneurones only after inflammation. The experiments presented in the present thesis are not able to resolve the problem but they caution about the interpretation of in vivo experiments.

The role of NK_1 and NK_2 receptors on synaptic transmission from dorsal roots to motoneurones.

It is well established that the fast monosynaptic and polysynaptic potentials (which usually last 50-200 ms) in the spinal cord and in particular on motoneurones are mediated mainly by AMPA and NMDA glutamate receptors (Jahr and Yoshioka, 1986; Ziskind-Conhaim, 1990; Jiang et al, 1990; Konnerth et al, 1990). Much less is known about long lasting (several seconds) polysynaptic potentials of motoneurones. They were strongly suppressed by NMDA receptor antagonists (Thompson et al, 1990) and it was suggested that tachykinins were contribuiting to slow potentials (Otsuka and Yoshioka, 1993). These suggestions were based on observations on dorsal horn cells (Urban and Randic, 1984) and on extracellular recordings from ventral roots (Woodley and Kendig, 1991; Gibbs and Kendig, 1991; Nagy et al, 1994; Thompson et al, 1994). Since the synaptic circuitry of the spinal cord is very complex, it is difficult to extrapolate the role of a particular receptor from dorsal horn to ventral horn. In the case of extracellular recordings from ventral roots change in cable properties of the axons could mask or enlarge the real effect. Moreover, in two cases (Nagy et al, 1994, Thompson et al, 1994) the antagonists used during experiments (RP 67580 and CP 96345) were found ineffective during the experiments presented in these thesis (CP 99994, the antagonist used in present thesis, is a modified version of CP 96345). Hence, the present experiments allowed to evaluate more thoroughly the neurotransmitter function of

substance P in contributing to postsynaptic potentials on motoneurones (even though studies with antagonists have limitations such as specificity and potency problems).

First, the surprising result was the lack of any clear effect of the NK_2 antagonist SR 48968 on postsynaptic potentials on motoneurones. It seems in contrast with the fact that the specific agonist for NK_2 receptor induced responses were selectively reduced (by 50%) by SR 48968 indicating the presence of NK_2 SR 48968 sensitive receptors in the spinal cord. Moreover, some studies have suggested the involvment of NK2 receptors in nociceptive sensation processing (Picard et al, 1993). The absence of the effects of SR 48968 on synaptic potentials could be explained by extrasynaptic receptor localisation: the receptors are far from the transmitter release site and, therefore, would be activated slowly. Nevertheless, it is unlikely as NKA-like (NKA is endogenous agonist for NK_2 receptors) immunoreactivity is detected within the first minute after a noxious stimulus in a large area of the dorsal horn of the cat spinal cord (Duggan et al, 1990) while in the present experiments no effect of SR 48968 even on long lasting (>30 s) tails following 20 s 1 Hz pulse trains was detectable (Fig. 17). On the other hand, the same studies (Duggan et al, 1990; Picard et al, 1993) provide a possible explanation for negative results, namely, that NK_2 receptor action could be extremely slow in onset (as the NKA-like immunoreactivity persists for 30 minutes and the effect of NK_2 agonist induced sensitisation lasted more than the one induced by NK_1 receptor agonist) and therefore will undetectable even through the 20 s long trains. Moreover, NK_2 receptors could act via other pathways distinct from those from dorsal roots to motoneurones (as it was shown that in dorsal horn cells NK_2. receptor antagonist supresses noxious heat induced responses only, Fleetwood-Walker et al, 1993). An alternative explanation is that there are several subtypes of NK2 receptor in the spinal cord and that the SR 48968 sensitive one is not important for synaptic transmission from dorsal roots to

motoneurones and these receptors could become inolved only after inflammation as suggested by Thompson et al (1994).

Second, the experiments with the NK_1 receptor antagonist SR 140333 confirmed previous suggestions that NK_1 receptors could participate in slow postsynaptic potentials evoked by small diameter fiber stimulation. The advantages of intracellular recordings allowed us to reveal several important aspects of NK_1 receptor function. First, in some cells the NK_1 antagonist reduced background spontaneous activity. This indicates that there were tonically active NK_1 receptors (presumably on interneurones) which modulated release of fast neurotransmitters (such as glutamate and GABA) via changes in membrane potential of interneurones. Traditionally substance P is supposed to mediate/modulate slow synptic potentials (Otsuka and Yoshioka, 1993): activation of G protein-coupled receptors (NK_1 receptors belong to G protein coupled receptor family) result in membrane potential changes (detected as postsynaptic potentials) via several intermediate steps which require some time to develop (>50 ms, Hille, 1994). This delay (>50 ms) is incompatible with the delays of the peaks of fast synaptic potentials (usually <50 ms, see Fig. 15) ruling out a function of substance P as fast excitatory transmitter on motoneurones. Nevertheless, tonically active NK_1 receptors could facilitate induction of spikes in interneurones (via input resistance increase or depolarisation of membrane potential, Murase et al, 1989) during dorsal root stimulation. Consequently, tachykinin receptor activation could increase the number of interneurones (hence, the amount of the released transmitter) involved in the generation of polysynaptic postsynaptic potentials. The same argument holds for spontaneous synaptic potentials. Such explanation is supported by the fact that the first peak of fast postsynaptic potentials (presumably, monosynaptic and, therefore, not dependent on interneurone activity) was unaffected by SR 140333 application. In addition, lack of change in the first peak indicates that SR 140333 did not affect directly the release of neurotransmitters via

nonspecific pathways (calcium channel block, etc). The second important observation is the independence of the action of SR 140333 from the presence of NMDA receptor antagonists. It is an important observation as most of NK_1 receptor effects on synaptic transmission in spinal cord are supposed to be due to modulation of NMDA receptor mediated responses (Urban et al, 1994). Even though the data presented in this thesis do not exclude a physiological role for NMDA receptor modulation (as observed in other laboratories, Rusin et al, 1993), the existence of an independent NK_1 receptor mediated component of slow synaptic potentials is strongly suggested. It should be noted that the data do not support the possibility of direct release of tachykinins from primary fibers as co-application of NMDA and non-NMDA glutamate receptor antagonists blocked both fast and slow postsynaptic potentials. It suggests two possible scenarios (not mutually exclusive): glutamate ionotropic receptor activation on interneurons leads to tachykinin release on motoneurone or alternatively the activation of tachykinin receptors on interneurons leads to glutamate release on motoneurones. Of course, there can be glutamatergic neurons activated by glutamate release from primary afferent fibers also. Hence, the independence of NK_1 receptor dependent component from the presence of NMDA and non-NMDA receptor antagonists suggests the existance of a separate peptidergic synaptic pathway (which includes glutamatergic interneurones or/and only glutamate releasing fibers). This independence of the action of NK_1 antagonist is further demonstrated with the analysis of the experiments with stimulus trains.

Role of NK_1 and NMDA receptors in the cumulative depolarization and its rate of rise

It is likely that the long duration of tachykinin receptor induced responses and, consequently, their mediated synaptic potentials could be important for summation of synaptic potentials. The cumulative depolarisation induced by 1 Hz trains (which can be accompanied by action potential windup, an important phenomenon for investigating the central components of nociception, Mendell and Wall, 1965) could be one example of such a complex summation (Thompson et al, 1990). While the nature of the cumulative depolarisation remains obscure, several observations from other groups (Thompson et al, 1990, Sivilotti et al, 1993) are relevant to our discussion:

first, there is no obvious relationship between the appearance of large cumulative depolarisation and the parameters of the single slow postsynaptic potential (except a requirement for >3 s duration of slow potentials); this indicates that cumulative depolarisation is not simple summation of synaptic potentials;

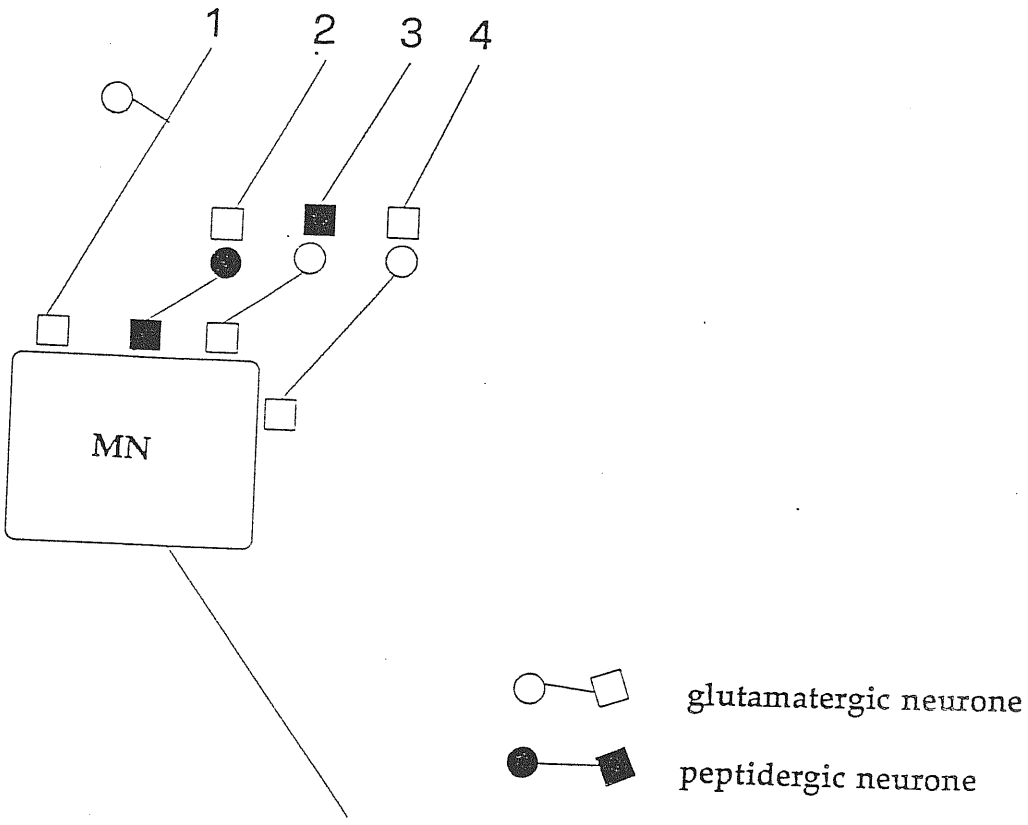
second, NMDA receptor antagonists strongly reduce cumulative depolarisation;

third, the rate of rise of cumulative depolarisation, in other words, the speed of summation, can predict the appearance of action potential windup, thus the rate of rise becomes an extremely important parameter of the cumulative depolarisation.

In addition, it has been concluded that the rise of extracellular potassium concentration due to high spiking activity could not account for such large depolarisations (Sivilotti et al, 1993).

On the basis of the first two observations it was suggested that the modulation of NMDA receptors by tachykinins could be responsible for cumulative depolarisation and high rate of rise (Urban et al, 1993). Nevertheless, the experiments presented in this thesis show that the rate of rise of cumulative depolarisation persists in the presence of NMDA receptor

Fig. Oversimplified scheme of some possible excitatory pathways activated by dorsal root stimulation and converging on spinal motoneurone (MN). Pathway 1 indicates monosynaptic connections from a primary afferent fibre (open circle indicates idealized cell body of this cell). Pathways 2-4 also originate from primary afferents but establish polysysnaptic connections inside spinal cord grey matter; the most elementary pattern of such connections with various combinations of glutamatergic and peptidergic synapses is shown. For simplicity inhibitory pathways were omitted. Open symbols depict glutamatergic cells while filled ones indicate peptidergic neurons.


1
2
3
4
MN
glutamatergic neurone
peptidergic neurone

antagonists (actually it is slightly increased). It is perhaps possible to explain this observation by suggesting that the cumulative depolarisation comprises, in fact, several, relatively independent components and its rate of rise represents the NMDA receptor independent component. In this case the reduction in the peak of cumulative depolarisation by NMDA antagonists might reflect the large sensitivity of the upstroke (i.e. the initial amplitude of cumulative depolarisation, see methods) to these antagonists since the peak is the sum of the upstroke plus the integral of rate of rise over the duration of train (the speed multiplied by the time gives the value of the trajectory, mV/s * s = mV, see methods). Indeed, in the presence of NMDA antagonists the upstroke was reduced by 79 % while the peak only by 40 % (the rate of rise was increased by 20 %). The additional evidence for the existence of independent components of cumulative depolarisation is that the NK_1 receptor antagonist SR 140333 reduced the peak mainly via a reduction in rate of rise: while the upstroke was almost unchanged (95 % of control value) the rate of rise was reduced to 67% and the peak to 71%. Moreover, SR 140333 was similarly effective in the presence of NMDA receptor antagonists: under these conditions SR 140333 reduced the rate of rise to 77 % of the value in the presence of NMDA receptor antagonist (this is, within the range of experimental error, similar to the result in control solution). In favour of the several component hypothesis are also the data on the voltage dependence of cumulative depolarisation parameters: while the upstroke decreased with depolarisation (the usual voltage dependence for fast postsynaptic potentials, Yoshimura and Jessel, 1990), the rate of rise increased during membrane depolarisation, a phenomenon which persisted in the presence of NMDA receptor antagonists, and the peak had intermediate voltage dependence (Fig.20, 21).

In summary then, the cumulative depolarisation could be represented as a sum of two parts:

the first one is NMDA receptor independent and it possesses the unusual voltage dependence, ~30% of it is due to NK_1 receptor. The response to a stimulus train in the presence of NMDA receptor antagonists mainly represents this component;

the second component is NMDA receptor dependent and NK_1 receptor independent and it posseses the usual voltage dependence; this component can be visualised by substracting the response in NMDA solution from the response in the presence of NMDA receptor antagonists, or by reduction in stimulus strength (see Fig. 23C). In this case the response is almost completely suppressed by NMDA receptor antagonists (Fig. 23B).

The second component lacks a well known NMDA receptor induced response feature,- namely its voltage dependent block: such a discrepancy could be explained by the quite low magnesium concentration at synaptic level or, more likely, by the fact that NMDA receptors are located mainly on interneurones (which release glutamate on motoneurone inducing the activation of nonNMDA glutamate receptors on motoneurones).

It should be noted that such a simple division is not applicable to the afterdepolarisation as its area was reduced significantly by both antagonists: by 37% by NMDA receptor antagonists and by 39% by SR 140333. Moreover, the sum of these two antagonisms corresponds to ~80% reduction, in other words, co-application of SR 140333 and NMDA receptor antagonist should result in an almost complete block of the afterdepolarisation. Nevertheless, such co-application suppressed the area of afterdepolarisation by 49% only. Furthemore, the antagonism by SR 140333 towards the afterdepolarisation was reduced in the presence of the NMDA receptor antagonists (from 39% reduction in control solution to 22% reduction in the presence of NMDA receptor antagonists). Hence, there was no simple summation of the effects indicating interaction between the two receptor systems (possibly, via modulation of NMDA receptor induced responses by tachykinins as it was suggested by Urban et al, 1994; alternatively, by interaction at network level).

The additional, important observation is that the cumulative depolarisation was completely blocked by co-application of NMDA and non-NMDA receptor antagonist. Hence, all components of cumulative depolarisation require glutamate ionotropic receptor activation (at least at one point in the polysynaptic pathway).

In conclusion, the present results indicate that NMDA receptors play a minimal role in the determining the rate of rise, the most important parameter of cumulative depolarisation for predicting action potential windup. Hence, another hypothesis for the nature of cumulative depolarisation was addressed.

Role of voltage dependent conductances in cumulative depolarization

In addition to the NMDA and NK1 receptor hypothesis there are other possible explanations for the rate of rise of cumulative depolarisation. One of them is based on the complex kinetics of calcium channels (Russo and Hounsgaard, 1994). According to studies of turtle spinal neurones repetitive stimuli progressively recruit slow Ca^{2+} channels which can mediate persistent excitation (Russo and Hounsgaard, 1994). In the simplest scheme for channel activation, the ability to turn on these channels would depend on how long a depolarizing synaptic signal (and its associated spiking) could persist above the activation threshold (Colquhoun and Hawkes, 1995). An analogous scenario seems unlikely for rat spinal motoneurones because in the present experiments after a ten fold decrease in postsynaptic potential area the rate of rise of cumulative depolarization was even larger than in control and had no correlation with the area of slow depolarization or total number of spikes in the train. A role for slow Ca^{2+} channels might therefore be surmised only by assuming complex kinetics of activation and inactivation, which are beyond the resolution of the present study and for which evidence is currently lacking. These observations also raise some interesting issues concerning the synaptic input required to express a high rate of rise

and windup. In fact, while a pulse must be sufficient to elicit a slow, long (~10 s) postsynaptic potential, there is little correlation of the rate of rise with the amplitude and duration of the slow postsynaptic potential (Sivilotti et al, 1993). In the present experiments, after reduction in area of the slow postsynaptic potential by CCP, a small increment in pulse strength generated high rate of rise of cumulative depolarization and action potential windup while the area of slow postsynaptic potential was actually about 1/3rd of the control one. Hence, in order to observe a high rate of rise and a windup of action potentials, an important characteristic is the pulse intensity (which presumably determines the type and/or quantity of neurotransmitter release by activating a certain class of fibers) rather than the mere size of the slow postsynaptic potential.

Slow depolarizing potentials (partially mediated by NK1 receptors) are responsible for the rate of rise of cumulative depolarization

Since the role of NMDA receptors and of voltage activated intrisic conductances in the rate of rise of cumulative depolarization was minimal, it is suggested that the rate of rise was determined by summation of slow potentials. These slow potentials can be partly revealed by CPP or APV which eliminated most background activity. In these conditions the slow potentials were observable in a short time window at 600-800 ms after stimulus (in the following text they will be termed slow tails). In control medium they were usually hidden by NMDA receptor dependent postsynaptic potentials. In support of the hypothesis of summation of these slow potentials there are the following findings: a), a positive correlation between the size of rate of rise and of slow tails, b) slow tails possessed the same voltage-dependence as rate of rise; c) both slow tails and rate of rise were depressed to the same degree by SR 140333. Perhaps their small size in a number of cells made them insufficient to

summate: this suggestion might provide an explanation for the expression of action potential windup by a fraction of neurones only. It explains also the lack of correlation between parameters of single postsynaptic potentials and the rate of rise. In fact, the size and duration (parameters which determine their summation speed) of these slow potentials is undetectable in control solution because of the background activity and probably do not correlate with the size of whole postsynapic potential. Even in the presence of NMDA receptor antagonists we can only guess the actual duration of these slow potentials, the variability of which could explain a relatively low correlation ($r = 0.75$) between the amplitude of slow potentials and the rate of rise. The suggestion that only a small part of slow postsynaptic potentials was responsible for summation implies that the other components of the postsynaptic potentials are not summating even though they were long enough (~10 s while the stimuli were delivered at 1 Hz). Hence these components presumably decremented during the train. This notion is supported by observation that the area of postsynaptic potentials was gradually reduced at the end of the train. Moreover, in the presence of NMDA receptor antagonists the reduction in the area of individual synaptic potentials was largely prevented, implying that the NMDA receptor independent part was less frequency dependent and, thus, more suitable for summation (in agreement with the fact that rate of rise, otherwise a speed of summation, was independent from NMDA receptors). What is the nature of such a reduction in the area of slow synaptic potentials? One clue is given by the fact that a single 1ms stimulus (able to produce ~3 s long postsynaptic potential in the control conditions) strongly suppressed spontaneous synaptic activity (induced , for instance, by NK3 receptor agonist senktide, see Fig. 24) for several seconds. The likely explanation of this fact is that strong 1 ms stimulus induced a long lasting premotoneuronal inhibition (perhaps similar to the one observed in dorsal horn cells, Jeftinija and Urban, 1994). Such inhibitory processes could explain why a large area of a single postsynaptic potential does not guarantee a high rate of rise in the train.

The prevention of the process by NMDA receptor antagonists suggests that inhibitory interneurons were driven by NMDA receptor activity. Of course, the present data is not enough to make any final conclusion about the precise mechanism of cumulative depolarisation but provides useful leads for future experiments.

The present investigation can outline only one of the transmitter mechanisms underlying slow postsynaptic potentials in the spinal cord. On the basis of previous observations and of the current data concerning the block by SR 140333, it seems that substance P (or another closely related endogenous tachykinin) contributed to about $1/3^{rd}$ of these slow potentials. The identity of the other (predominant) mechanisms remains obscure: a role for other tachykinin receptors is not precluded since further tests will have to be carried out with antagonists against other NK receptor subclasses. Nevertheless, the hypothesis of summation of slow synaptic potentials independently from glutamate receptor activation suggests the exclusive role of these slow potentials and, consequently, of their neurotransmitters in synaptic integration of nociceptive signals. Hence, substance P can be one of the neurotransmitters required for induction of pain induced sensitisation. The specific action of NK_1 antagonist SR 140333 could be useful for the elaboration of pain relieving drugs.

The voltage dependence of responses to substance P

The action of substance P on spinal neurones is known to be voltage-dependent as it is enhanced by depolarization (Urban and Randic, 1984): this property may be in part the reason for the increase in slow EPSP amplitude with depolarization and it would impart a degree of voltage-dependence to the rate of rise. On the other hand, the SR 140333 -induced block of rate of rise or slow tails was not voltage dependent as it remained proportionally the same at two different levels of membrane potential, indicating that this agent was removing

the same fraction of NK1 receptor-mediated response contributing to the rate of rise. There are two possible explanations for the voltage dependence of responses to substance P. First, the closure of a conductance which is active at the rest potentials. Thus, this conductance should determine partially the resting potential and the reversal potential of this current should be negative to the resting potential in the case of depolarising responses. Second, substance P could activate voltage gated channels opened by depolarisation. In this case the reversal potential of the current should be positive to the resting potential in the case of depolarising responses. In both cases the result will be similar: the responses will grow with depolarisation and the increase of input resistance will be observed. It is possible that both mechanisms will work in the same cell as tachykinins can modulate several conductances (Murase et al, 1989; Shen and North, 1992). The DC tests during substance P responses (see Fig. 2) showed that the increase in input resistance is larger at depolarised membrane potentials than at the resting membrane potential. It clearly suggests that the current underlying substance P responses was voltage dependent. Hence, the second mechanism was more likely or the prevailing one. In contrast, from experiments with hypoglossal motoneurones it seems that the first explanation was more likely as the reversal potential was negative to resting potential and the substance P induced current seemed not to have any strong voltage dependence (Fig. 25C). This result suggests that the reduction in leakage conductance could be responsible for the response. Ionic substituition experiments might resolve the problem but the scarceness of substance P responsive cells did not allow to address the question properly. It might be due to immaturity of the preparation (as in the case of TRH induced responses in hypoglossal neurones, Bayliss et all, 1994), possibly due to the immaturity of the second messenger system or of the channels which are modulated by tachykinin receptors.

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