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An electrophysiological study of the effects of substance P on CA1 neurones of the mouse hippocampal slice preparation.

Thesis submitted for the degree of "Doctor Philosophiae"

Biophysics Sector

candidate
Maria Kouznetsova

supervisor
Prof. Andrea Nistri

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NOTE

This work was carried out at the International School for Advanced Studies (S.I.S.S.A., Trieste, Italy) and has not been submitted in whole or in part to any other University. The candidate personally performed all experiments, data analysis and contributed to paper writing. Part of the data reported in this thesis have been published in the article mentioned below.

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ABBREVIATIONS

ACSF- artificial cerebrospinal fluid

ACh- acethylcholine

AHP- afterhyperpolarization

AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APV- *D*-2-amino-5-phosphonopentanoic acid

CA1-4- zones of Cornus Ammon

CCh- carbachol chloride

CNQX- 6-cyano-7-nitroquinoxaline-2,3-dione

EPSP- excitatory postsynaptic potentials

EC- entorhinal cortex

GABA- γ-Amino-butyric acid

IPSP- inhibitory postsynaptic potential

mGluR- metabotropic glutamate receptor

NK- neurokinin

NKA, NKB- neurokinin A, neurokinin B

NMDA- *N*-methyl-*D*-aspartate

NPK, NPγ- neuropeptide K, neuropeptide γ

PPT- preprotachykinin

PT- protachykinin

RMP- resting membrane potential

sEPSP(s) slow cholinergic EPSP(s)

SP- substance P

SPME- substance P methylester

SR140333- ((*S*)-1-[2[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenyl)-piperidin-3-ethyl]-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride)

SML- stratum lacunosum moleculare

SO- stratum oriens

SR- stratum radiatum

StP- stratum pyramidale

TTX- tetrodotoxin

ABSTRACT

Substance P (SP), a neuropeptide that belongs to the tachykinin family, is widely distributed of the mammalian hippocampus together with its binding sites. In this particular brain region in the rat SP has been implied in early neuronal development, facilitation of learning and seizure activity (Huston and Hasenohrl, 1995; Sprick *et al.*, 1996, Taoka *et al.*, 1996; Sperk *et al.*, 1990). Up to now no systematic electrophysiological analysis has been undertaken to characterise its cellular effects on hippocampal neurones unlike for example the work done on the spinal cord, where its neurotransmitter role is well established (for review Otsuka and Yoshioka, 1993).

In the present study the effects produced by bath application of SP (2-4 μM) or the selective NK_1 receptor agonist substance P methylester (SPME; 10 nM-5 μM) were investigated employing intra- or extracellular recordings from the CA1 region of the mouse hippocampal slice preparation. Field potentials evoked by focal electrical stimulation of Schaffer collaterals and recorded from stratum pyramidale were depressed in the presence of the neuropeptide. This effect was NK_1 receptor mediated as it was completely blocked by the selective NK_1 antagonist SR140333, and it required an intact GABAergic network drive. The evoked excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) recorded from CA1 pyramidal neurones were similarly depressed by SPME. At the same time this neuropeptide increased the frequency (but not the size) of spontaneous GABAergic events and reduced the frequency of spontaneous glutamatergic events. Both effects of SPME on spontaneous and evoked IPSPs were prevented by the ionotropic glutamate receptor blocker kynurenic acid. No changes in frequency or size of spontaneous events produced by SPME were evident in

TTX solution. The responses of pyramidal cells to 4 μM AMPA or 10 μM NMDA were not altered by SPME.

To investigate the modulatory effect of SPME on cholinergic synaptic transmission in the hippocampus, brief (1-2 min) application of CCh (0.1 μM-100 μM) or electrical stimulation at the stratum oriens/alveus border was utilized. The observed excitatory response consisted of membrane potential depolarization, input resistance increase (or no change), bicuculline-sensitive repetitive oscillatory activity and slow synchronized events, which were supposed to be mediated via M1 muscarinic receptor subtype, as they were reversibly blocked by pirenzepine and by atropine in irreversible manner. All the components of this cholinergic response were potentiated in a dose dependent and SR 140333 sensitive manner by pre-application of SPME. The enhancement by SPME of depolarization produced by CCh application was present (albeit attenuated) in TTX solution. By itself SPME produced minimal changes in passive membrane properties of CA1 pyramidal cells, which were unable to account for the observed effects on fast and slow synaptic transmission.

The present data indicate that on CA1 pyramidal neurones SPME exerted its action via a complex network mechanism, which presumably involved facilitation of a population of GABA-ergic interneurones widely interconnected with excitatory and inhibitory cells in this hippocampal region.

INTRODUCTION

1.1. SUBSTANCE P AND THE FAMILY OF MAMMALIAN TACHYKININS

Substance P (SP) was the first peptide discovered in the neural tissue (Maggio, 1988). It was for the first time described by von Euler and Gaddum in 1931 as a substance (extracted horse brain and intestine) "which lowers blood pressure in atropinized rabbits by peripheral vasodilatation and also stimulates the tone and rhythm of the rabbit isolated intestine" (von Euler and Gaddum, 1931). The name SP came from "powder", the form in which extract of horse brain and intestine was prepared for those experiments. Only fourty years later SP was purified from bovine hypothalamus, sequenced and synthesised (Chang et *al.*,1971; Tregear et *al.*,1971).

Erspamer (1981) included SP into a large peptide family, which he called tachykinins (literally, fast acting) according to their pharmacological activity. This family comprises structurally related bioactive decapeptides with the common COOH- terminal sequence of -Phe-X(Phe, Tyr, Val, Ile)-Gly-Leu-Met-NH_2 derived from mammalian and non-mammalian tissues. Today, according to the nomenclature adopted at the Montreal Symposium (Henry et *al.*, 1987), the term "tachykinins" is used to indicate non-mammalian peptides such as kassinin, physalaemin, eledoisin. Mammalian peptides are designed as "neurokinins" (NKs) and the most studied among them are SP, neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK), neuropeptide γ (NPγ).

1.1.1. NK biosynthesis

Unlike classical neurotransmitters, which are synthesised by enzymes in nerve terminals, NKs are synthesised ribosomally as large precursors (pretachykinins, PT) in the cell

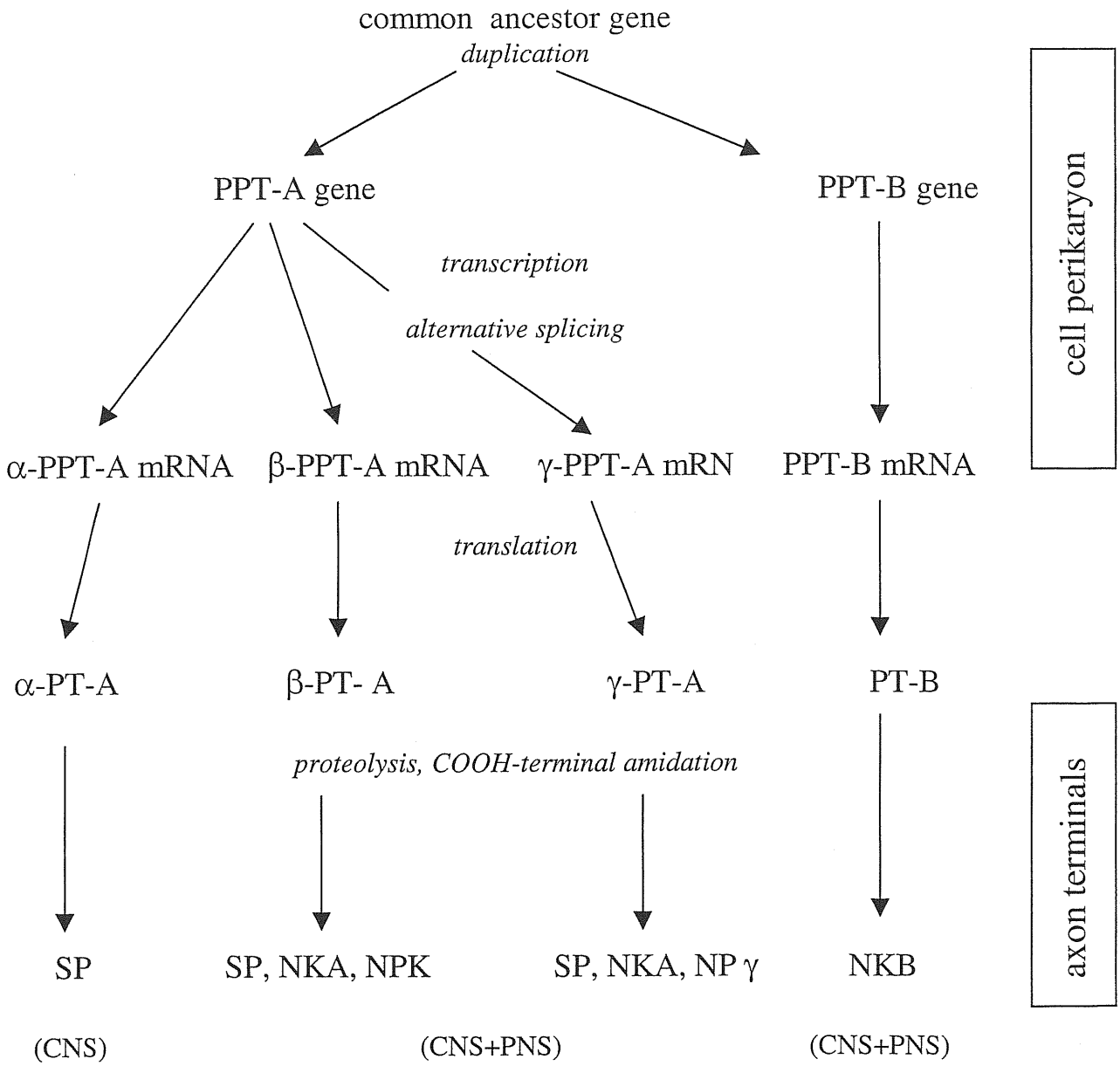

Fig. 1. Schematic representation of neurokinin biosynthesis. (from Kotani et *al.*, 1986; Nakanishi, 1987; Nawa et *al.*, 1990; Nakanishi, 1993; PTT-preprothachykinin, PT-protachykinin);

perikaryon and are then transported to the nerve terminals (for review see Otsuka and Yoshioka, 1993; Regoli et *al.*, 1994). The schematic representation of the process of biosynthesis and maturation of NKs is depicted in Fig.1 using data from Nakanishi's group (Kotani et *al.*, 1986; Nakanishi, 1987; Nawa et *al.*, 1990; Nakanishi, 1993). Thus, it seems probable that NKs could be released and acting far from the place of their synthesis.

1.1.2. NK distribution

NKs are widely present throughout the mammalian central nervous system (CNS) and in peripheral tissues. Different patterns of distribution for three main NKs (as well as for their precursors and mRNAs in mammalian tissues) have been discovered by employing radioimmunoassay and immunohistochemical studies. In the CNS SP-immunoreactive cell bodies and fibres are organised into a kind of network with its intrinsic and exogenous projections (see Fig. 2). Among the regions most heavily supplied with SP are substantia nigra, striatum, intrapeduncular nucleus, habenula, nucleus of the tractus solitarius, hypothalamus, raphe nucleus, medulla oblongata and spinal cord, especially the substantia gelatinosa of the dorsal horn (see Table 1 from Otsuka and Yoshioka, 1993). NKA has analogous distribution in the CNS and in the periphery. SP and NKA are often co-localised within the same cells, although the amount of NKA is generally smaller than SP. Unlike SP/NKA, NKB is detected in different regions of the CNS (see Table 1). In the periphery NK containing fibres are either axons of primary afferent neurones, whose cell bodies are in spinal dorsal root ganglia and in cranial sensory ganglia, or autonomic

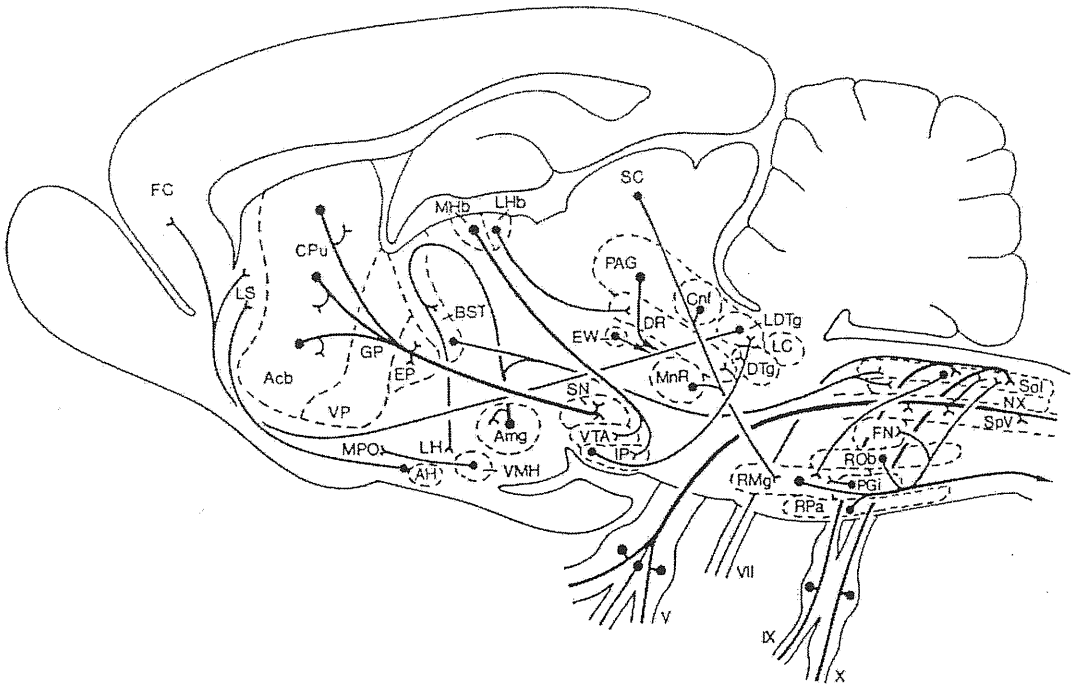

Fig. 2. Localization of SP-containing cell groups and fibres in mammalian CNS. (sagittal section of the brain; the most data derived from the rat). From Otsuka and Yoshioka, 1993.

TABLE 1

Distribution of tachykinins and tachykinin receptors in the CNS and PNS of the rat (from Otsuka ans Yoshioka, 1993).

REGIONS TACHYKININS (pmol/g) RECEPTORS

	SP	NKA	NKB	NK_1	NK_2	NK_3
Cerebral cortex	7	4	2.9	-	-*	+++
Striatum	312.2	26.2	1.2	++++	-	-
Globus pallidus	114.7	68.9	8.3			
Substantia nigra	1154.2	115	2.8	-	-	-
Ventral tegmental area	115.4	68.9	17.2	-	-*	+
Nucleus accumbens	122.9	70.4		+++	-	-
Hippocampus	4.1	3	1.1	++	-*	+
Hypothalamus	201.4	171.3	31.4	+	-	-
Habenula	406	240.2		++	-*	+
Interpeduncular nucleus	474.6	168.3		++	-*	++
Cerebellum	4.7	1.6	1.6	++	-	-
Solitary nucleus	459	389.2	21.7	+++	-*	++++
Raphe nuclei	276	83		+	-	-
Medulla oblongata	226.4	71.1	7.2	++	-	-
Spinal cord:						
dorsal horn	503.7	65.9	9.1	++	-*	++++
intermediolateral nucleus	121.6	126	31.6	++++	-	-
ventral horn	117.4	16.1	2	++	-	-
Ileum	47.8	23.9	0.44	+++	++	+
Colon	20.1	16.1	0.67	++++	++++	+
Parotid gland	16.2	0.4	0.4	+++	-	-
Submandibular gland	12.5	5	0.5	++++	-	-
Heart	0.4	0.2	0.3	-	-	-
Urinary bladder	1.7	3.6	0.3	++++	++++	+
Adrenal grand	1.2	0.8	1	-	++++	-
Skin	2.5			+	-	-

* in these regions NKA binding sites were detected by autoradiography, whereas NK_2 receptor mRNA was undetectable in CNS by blot hybridization.

ganglion axons. Finally, there is a population of NK-positive neurones in the retina and even in some non-neuronal cells, for example human eosinophils and enterochromaffin cells (for review see Otsuka and Yoshioka, 1993 and references therein).

1.1.3. NK co-localisation

In some regions of the mammalian nervous system NKs are known to coexist with other peptides and/or classical neurotransmitters (Hokfelt, 1991). Such a co-localisation was first noted for SP and serotonin (5-HT) in brain stem neurones and their fibres which project to ventral horn motoneurones (Hokfelt et *al.*, 1978). Most examples of co-localisation were found in the spinal cord, for instance, in primary sensory neurones where SP co-exists with CGRP and glutamate to modulate the nociceptive flexor reflex (Woolf and Wiesenfeld-Hallin, 1986) and in the autonomic (enteric) nervous system (Hokfelt, 1991).

In the mammalian cerebral cortex, for example in the primate prefrontal cortex, electron microscopic studies using double label immunocytochemical techniques revealed that all SP-immunoreactive boutons throughout all layers contain GABA. Moreover, in the same brain region these SP-positive axon terminals form symmetric synapses on the dendritic spines where they are targeted by asymmetric glutamatergic boutons, establishing so called "SP-ergic synaptic triads" (Jakab et *al.*, 1997). The functional significance of such SP co-localisation (as well as the coexistence of any other peptide with fast neurotransmitters) could be the fine tuning and precise programming of the synaptic transmission by modulation of transmitter release mechanisms, time course of the responses and distribution of targets (Otsuka and Yoshioka, 1993).

1.1.4. NK receptors

a) Taxonomy

According to the Montreal classification (Henry, 1987) three types of NK receptors (NK) can be distinguished on the basis of the potency order of the naturally occurring mammalian tachykinins. They are termed as NK_1, NK_2 and NK_3. It is important to stress that all known endogenous NKs can act as full agonists at all three types of receptors, although with different affinity (Regoli et *al*, 1994). The agonist order of potency is the following:

for NK_1 receptor- SP>NKA>=NKB

for NK_2 receptor- NKA>NKB>>SP

for NK_3 receptor- NKB>NKA>>SP

The existence of distinct receptor types was proved by molecular cloning and they were functionally characterised in electrophysiological experiments (Harada et al., 1987; Nakanishi et al., 1990).

b) Properties

NK receptors belong to the family of G-protein coupled rhodopsin-like receptors (Regoli et *al.*, 1994). Comparative analysis of the three receptor types revealed a high degree of homology (54-66%) in all seven trans-membrane domains as well as in the cytoplasmic tail (Nakanishi, 1991).

The biological actions mediated by NK_1, NK_2 and NK_3 receptors are supposed to be evoked by the C-terminal sequence of the tachykinins (Maggi et *al.*, 1993).

In most tissues (and cell lines transfected with cDNA encoding for a certain receptor subclass) all three NK receptor subtypes are coupled to the pertussis toxin-insensitive G_q/G_{11} protein family, which is linked to phosphoinositide metabolism via activation of phospholipase C (Mantyh et *al.*, 1984; for review see Otsuka and Yoshioka, 1993; Khawaja and Rogers, 1996). Intracellular metabolites from phosphoinositol breakdown triggered by NK receptor activation, release Ca^{2+} from intracellular stores which in turn activates protein kinase C to open voltage-gated Ca^{2+} channels (Gallacher et al., 1990): this series of events is responsible for SP-induced actions such as smooth muscle contraction (Watson, 1984; Tachado et al., 1991) or secretion from salivary gland cells (Laniyonu et al., 1988). In nerve cells the stimulation of the phosphatidylinositol-Ca^{2+} system by NK_1 receptor activity leads to suppression of the inward rectifier K^+ current and increase in a non-selective cationic conductance (Nakajima et al., 1988; Koyano et al., 1993; Takano et al., 1995; Parsons et al., 1995; Bell et al., 1998; for details on SP effects in CNS see also further chapters).

Although phospholipase C activation is considered to be the principal effector for NK receptors, these are coupled to adenylate cyclase activation (presumably via the pertussis toxin-sensitive G_S protein) in certain smooth muscle cells and cell cultures transfected with NK receptors (Tachado et al., 1991; Mitsuhashi et al., 1992). Conversely, NK receptor activation is also reported to inhibit adenylate cyclase (via a pertussis toxin-sensitive G_I/G_o protein) in salivary gland cells (Laniyonu et al., 1988). The existence of multiple intracellular effectors coupled to different G proteins could explain the cell type specificity of the effects of SP. In some cells, for instance the submandibular acinar cells,

SP can simultaneously act via several separate transduction pathways (Laniyonu et al., 1988).

Like the majority of G protein-coupled receptors, NK receptors undergo homologous desensitisation after repeated or prolonged exposure to the agonist (Maggio, 1988). The rate of desensitisation differs among NK receptor types. As shown by Nakanishi and colleagues (1990) for rat NK receptors expressed in *Xenopus* oocytes, the NK_1 receptor type exhibits much stronger desensitisation than NK_2 or NK_3 receptors. Rapid endosomal internalization of the receptor (Mantyh et *al.*, 1995, but see Sanders and Le Vine Third, 1996) and phosphorylation of the serine-threonine containing regions (third intracellular loop and cytoplasmic C-terminal of NK_1 receptor) are thought to be responsible for the desensitisation induced by SP (Ohkubo and Nakanishi, 1991). Using chimeric receptors Sanders and Le Vine Third (1996) proved that NK_1 desensitisation is dependent on the presence of the carboxy-terminal region.

NKs produce a number of biological effects, such as release of histamine from mast cells, modulation of catecholamine release from adrenal medulla, GABA release from dorsal root ganglia, certain behavioral responses and analgesia (reviewed by Maggi et *al.*, 1993), that do not involve NK receptors and thus are thought to be due to their N-terminal fragment. Although some specific binding sites recognizing the N-terminal sequence of SP have been described in mouse brain (Igwe et al., 1990), direct activation of a pertussis toxin-sensitive G-protein is proposed, for example, for the receptor-independent mast cell degranulation induced by SP (reviewed by Maggi et al., 1993). The N-terminal sequence is also thought to play a role in ensuring the conformation changes of the COOH-terminal of NKs and their ability to distinguish various NK receptors (Cascieri et *al.*, 1992).

c) Distribution

Tachykinin receptors are differentially distributed in the mammalian CNS and in peripheral tissues (see Table 1). In the brain high levels of the SP binding sites and mRNA for NK_1 receptors were observed in striatum, olfactory bulb, hypothalamus and spinal cord, whereas the NKB receptor binding sites and NK_3 mRNA levels were noted to be higher in the cortex, cerebellum, hypothalamus and substantia nigra (Saffroy et *al.*, 1988; Tsuchida et al., 1990; Humpel and Saria, 1993; Shughrue et *a*l., 1996). The expression of NK_2 receptors appeared to be restricted mainly to peripheral tissues: urinary bladder, stomach, intestine, adrenal gland (Saffroy et *al.*, 1988; Tsuchida et *al.*, 1990; Humpel and Saria, 1993).

d) Mismatch between neurotransmitter and receptor localisation

In many areas of the mammalian nervous system there is a positive correlation between the density of NK containing fibres and their receptors, but in certain regions of the CNS a significant mismatch has been noted. For example, in the substantia nigra, substantia gelatinosa of the trigeminal tract nucleus and in the dorsal horn of the spinal cord there is high SP immunoreactivity but a low density of binding sites for SP and/or lack of NK_1 mRNA expressing cells (Mantyh et *al.*, 1984; Herkenham, 1987; Maeno et *al.*, 1993). One possible explanation for this mismatch could be the existence of unknown receptor subtypes in these CNS regions (Quirion et *al.*, 1983; Maeno et *al.*, 1993). Another plausible hypothesis is that the peptide acts at considerable distance from the site of its release (Agnati et *al.*, 1995). The idea of such "non-synaptic" or so called "volume transmission" brought about by SP is also supported by the electron microscopic study of

Liu and co-workers (1994), showing that in rat striatum, cortex and spinal cord SP receptors are not restricted to the synaptic region, but rather cover a large part of the neuronal somata and dendritic tree, thus making the whole cell surface a possible target for the neuropeptide. In agreement with the volume transmission theory are also the observations that SP in SP-containing terminals is stored in large granular vesicles and is maximally released upon high frequency electrical stimulation, features supposed to be characteristic of nonsynaptic transmitter release (Floor et. *al.*, 1982; Zhu et *al.*, 1986; Iverfeldt et *al.*, 1989). Unlike classical neurotransmitters SP is not subjected to subsequent neuronal re-uptake (for review Agnati et *al.*, 1995). Moreover, like many other neuropeptides, mammalian tachykinins after their release into the extracellular space undergo enzymatic cleavage with their fragments diffusing widely and persisting for a long time, as shown by Duggan et *al.* (1990) for NKA liberated by noxious stimulation from spinal cord and traced with antibody microprobes.

1.2.5. Biological effects and mechanism of NK action in the CNS

The hypothesis of a SP role as a neurotransmitter (initially as sensory neurotransmitter in the spinal cord) had been proposed by Lembeck in 1953 (Otsuka and Yoshioka, 1993) even before the structure of the peptide was defined and SP was discovered in small-diameter cell bodies of dorsal root ganglia and fine calibre axons in superficial dorsal horn (Hokfelt et *al.*, 1975).

Today, in addition to their role in sensory (mainly nociceptive) transmission, NKs are implied as neuromediators of such functions as motor activity, visceral and endocrine regulation, memory and learning.

A great deal of *in vivo* and *in vitro* electrophysiological studies revealed powerful excitatory effect of NKs on the CNS (for review see Otsuka and Yoshioka, 1993).

In different neuronal cells SP and other NKs applied exogenously, as well as released upon electrical or noxious stimulation, produce a slow membrane depolarization (or slow EPSP) that mainly accounts for the increase in cell excitability (Murase et *al.*, 1989; De Koninck and Henry, 1991; Shen and North, 1992; Aosaki and Kawaguchi, 1996). The ionic changes underlying this phenomena could be divided into two groups: a) decrease in K^+ conductance due to inhibition of the fast inward rectifier as shown on cholinergic forebrain neurones (Stanfield et *al.*, 1985; Aosaki and Kawaguchi, 1996; Bell et *al.*, 1998), brain stem auditory efferent neurones (Wang and Robertson, 1998), dorsal vagal neurones (Martini-Luccarini et *al.*, 1996) and neurones from locus coeruleus (Shen and North, 1992), or inhibition of the Ca^+-activated K^+ current or the M-current as described in sympathetic bullfrog ganglia (Adams et *al.*, 1983), or suppression of the current carried through potassium selective channels, contributing to the leakage current observed in the spinal cord motoneurones (Fisher and Nistri, 1993); b) increase in some non-selective cationic conductances as demonstrated in sympathetic ganglion neurones (Minota et *al.*, 1981), spinal cord dorsal horn neurones (Murase et *al.*, 1989) and rat locus coeruleus neurones (Shen and North, 1992). SP has also been shown to inhibit N-type Ca^{2+} currents in sympatheic neurones (Bley and Tsien, 1990) and to increase Ca^{2+} current in spinal dorsal horn neurones (Murase et *al.*, 1989). Thus, the ionic mechanisms operated by SP vary in different neuronal preparations and sometimes co-exist in the same cell (for example, in case of the locus coeruleus neurones; Shen and North, 1992). Indeed,

different changes in input resistance (an increase, a decrease or none) have been described associated with SP depolarization, implying involvement of diverse ionic conductances.

Raised intracellular Ca^{+} concentration, which has been reported as another effect produced by SP, has been attributed to the activation of voltage dependent Ca^{2+} channels by depolarisation, as well as to the inositol phospholipid hydrolysis via G-protein coupled NK receptors with IP_3 formation and subsequent Ca^{2+} release from the internal store (Otsuka and Yoshioka, 1993; Heath et *al.*, 1994).

All effects described so far are directly expressed by SP at postsynaptic level, as they persisted in the presence of TTX. There is evidence that NKs can also participate in the presynaptic modulation of neurotransmitter release. This was suggested by co-localisation of NKs with other neuropeptides and transmitters, and studied mainly in superfusion experiments. In fact, besides previously mentioned examples (see NK co-localisation chapter), SP is known to enhance the spontaneous release of dopamine and largely reduce the NMDA -evoked dopamine release from the striatum (Gauchy et *al.*, 1996). In the rat cerebral cortex SP increases basal and evoked release of 5HT and ACh (Solti and Bartfai, 1987; Sastry, 1995, but see Lamour et *al.*, 1983). SP causes inhibition of glutamate-mediated transmission in the pontine parabrachial nucleus (Saleh et *al.*, 1996), but on the other hand it releases excitatory amino acids in the dorsal spinal cord (Smullin et *al.*, 1990). SP-evoked release of GABA has been described in the spinal cord of the newborn rat (Sakuma et *al.*, 1991).

The mechanisms underlying such diverse effects of SP on transmitter release are not fully understood. An increase in intracellular Ca^{2+} levels is thought to enhance transmitter release and can be induced by transduction pathways activated via NK receptors.

Gallacher et al. (1990) have shown that in rat pancreatic acinar cells the breakdown of phosphatidylinositol-diphosphate by SP generates diacylglycerol to activate protein kinase C which increases Ca^{2+} influx via voltage-dependent Ca^{2+} channels. A direct effect of protein kinase C on the opening and number of functional, voltage-dependent Ca^{2+} channels has also been demonstrated (Dolphin, 1991 and references therein). The intracellular metabolite inositol-1,4,5-triphosphate *per se* can also facilitate Ca^{2+} entry across the cell membrane (Kuno and Gardner, 1987).

The NK_1-receptor mediated increase in a Ca^{2+} dependent K^+ conductance resulting in membrane hyperpolarisation, (described for instance in the ferret vagal sensory neurones by Jafri et *al.*, 1996), is proposed to be a possible mechanism for a presynaptic inhibitory action of the peptide.

In summary, the evidence for the neurotransmitter action of SP in the CNS comes from:

1) wide distribution of SP-immunoreactive cell bodies and SP-containing fibre networks (see Fig. 2); graded release of NKs in response to stimulation (for instance, Ca^{2+}dependent release of SP upon repetitive electrical stimulation of dorsal roots);
2) detection of distinct groups of NK receptors and their distribution mainly (with the exception of mismatch cases) in accordance with the distribution of NK-containing fibres;
3) direct actions (mainly excitatory) of NKs on postsynaptic targets, that are blocked by selective NK antagonists.

In some regions of the nervous system, especially where SP and NKs do not elicit measurable direct electrophysiological changes at the postsynaptic level, they affect the

release and effectiveness of classic neurotransmitters, thus fulfilling a neuromodulatory function.

1.1.6. NK agonists and antagonists

Naturally occurring NKs are non-selective as they activate, although with a different range of potency, all three types of the receptor. Thus, in order to study the structure and function of endogenous neuropeptides and to map NK receptors, selective agonists and antagonists for each type of receptors were synthesized. They mainly fall into two categories: peptidergic and non-peptidergic. The latter have several advantages because they are more stable, easier to synthesise and capable of penetrating through the blood brain barrier (reviewed by Maggi et al, 1993). The last aspect could be of great value to experiments *in vivo* and to therapeutic applications. The most frequently used compounds are listed in Table 2. Wide distribution and broad spectrum of NK biological activities have suggested that NK receptor ligands could be useful for the treatment of various clinical conditions. In particular, this application for NK antagonists is currently investigated for central and peripheral analgesia, headache, neurogenerative diseases (including Alzheimer's disease, multiple sclerosis, etc.). Since SP appears to play a pathogenetic role in allergic reaction, and neurogenic inflammation (asthma-bronchial hyperreactivity, rheumatoid arthritis, certain chronic diseases of gut), using NK antagonists for treatment of these human disorders has been discussed (see for review Maggi et *al.*, 1993).

TABLE 2 **Classification of tachykinin receptors** (from Trends in Neuroscience, 1992).

NOMENCLATURE	NK_1	NK_2	NK_3
Previous names	substance P	substance K	neurokinin B
	SP P	SP E/SP K	SP E/SP N
Potency order	SP>NKA>NKB	NKA>NKB>>SP	NKB>NKA>SP
Selective agonists	SP methyl ester	[β-Ala^8]NK-4-10	senktide
	[Sar^{9}Met$(O_2)^{11}$]SP	GR64349	[MePhe^7]NKB
	[Pro^9]SP	[Lys^{5}MeLeu^{9}Nle^{10}]NK-4-10	[Pro^7]NKB
Selective antagonists	CP99994 (K_i0.5-50 nM)	SR48968 (K_i 9 nM)	SR142802 (K_i9.2 nM)
	SR140333 (K_i0.02-0.5nM)	GR94800 (K_i 9.6 nM)	PD157672 (K_i16nM)
	LY303870 (K_i 9.4 nM)	MEN10627 (K_i8.6 nM)	
	RP67580 (K_i 3-12 nM)	GR159897 (K_i 9.5 nM)	
Radioligands	[^{3}H]or[^{125}I]BH[Sar^{9}Met$(O_2)^{11}$]-SP	[^{3}H]SR48968	[^{3}H]senktide
	[^{125}I]L703606	[^{3}H]GR100679	[^{3}H]SR142802
	[^{3}H]or[^{125}I]BH-SP	[^{125}I]NKA	[^{125}I][MePhe^7]NB
Gene	*nk1*; chr2	*nk2*; chr 10	*nk3*

CP99994- (+)-(2s,3s)-3-(2-methoxybenzylamino)-2-phenylpiperedin
GR64349- Lys-Asp-Ser-Phe-Val-Gly-[R-γ -lactam]
GR159897- 5-fluoro-3-ylethyl(4-[phenylsulphinyl methyl]) piperidines
L703606- (cis)-2(diphenylmethyl)-N-[(2-iodophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amide
LY303870- (R)-1-(N-[2-methoxybenzyl]acetylamino)-3-(1H-indol-3-yl)-2-(N-[2-{4-(piperidin-1-yl) piperidin-1-yl}acetyl]amino)propane
MEN10627- cyclo(Met-Asp-Trp-Phe-Dap-Leu-)cyclo(2β-5β)
PD157672- Boc(S)Phe(R)a MePheNH$((CH_2)_7$NHCON H_2
RP67580- (1-imino-2-(2-methoxy-phenyl)-ethyl-7,7-diphenyl-4-perhydroisoindolone(3aR,7aR)
SR48968- (s)-N-methyl-N-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butylbenzamide
SR140333 (s)-1-[2[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenyl)-piperidin-3-ethyl]-4-phenyl-1-azoniabicyclo[2.2.2]
SR142802-(s)-(N)-(1-(3-(1-benzoyl-3(3,4dichlorophenyl)pipreidin-3-yl)propyl)-4phenylpiperidin-4-yl)-N-methylacetamide

1.2. MAMMALIAN HIPPOCAMPUS

The hippocampus is a cylindrical structure located within the medial temporal lobe of the brain (see Fig. 3).

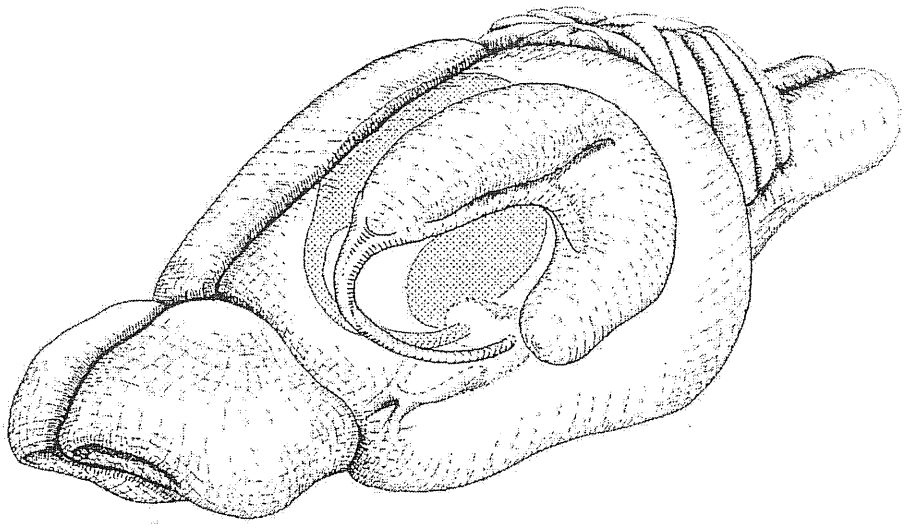

Fig. 3. The three-dimensional organization of the hippocampal formation in the rat brain. The both hippocampi are shown in a transparent shell of the brain. (from Paxinos, 1995)

There are several reasons for choosing this structure for investigating SP effects on the CNS. In the hippocampus there is an intermediate level of this neuropeptide together with its binding sites while a detailed description of its characteristic network organization has recently become available (Acsady et *al.*, 1997; Borhegyi and Leranth, 1997). The hippocampus is involved in normal cognition (for review see Squire, 1992; Jarrard, 1995 and Eichenbaum, 1997) as well as in some severe neurological disorders such as Alzheimer's disease and epilepsy in which SP and other neurokinins are proposed to play a role (Huston and Hasenohrl, 1995; Sprick et *al.*, 1996. Sperk et *al.*, 1990; Yankner et al., 1990). Furthermore, from an anatomical point of view this brain region is a

convenient model to address various aspects of neurotransmission because its cytoarchitecture remains relatively well preserved in the in vitro slice preparation (see Fig. 4).

Even if most information concerning NK pathways and receptor distribution comes from rat hippocampus, the present work was carried out on the mouse hippocampal slice preparation. Two reasons led to the choice of the latter animal species: 1) the present experiments were designed to lay the foundations for subsequent work on transgenic mice expressing neuroantibodies to SP; 2) in the mouse the organization of hippocampal CA1 area is very similar to the one found in the rat (Yanovsky *et al.*, 1997).

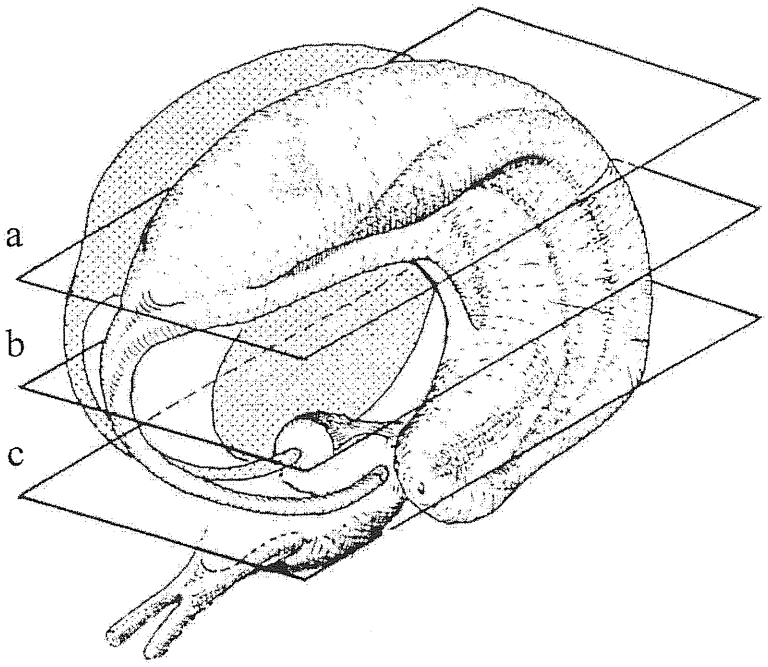

Fig. 4. The organization of the rat hippocampus. The horizontal sections at different levels of the hippocampal formation. Note that for present experiments medial section was used (marked as B). From Paxinos, 1992.

1.2.1. Hippocampal structure

It was Ramon y Cajal (1893) who first described the architecture of hippocampus. Although the nomenclature introduced by Ramon y Cajal is now obsolete, his famous original representation of the structure of hippocampus is still widely used today (see Fig.5). The hippocampus proper, according to Lorento de No' (1934), can be divided into four regions: CA1-4 (Cornus Ammonis). CA1 and CA3 usually are the most prominent zones, CA2 in some species is not very well developed and the region between dentate gyrus and CA3 originally termed CA4 is now known as the hilus.

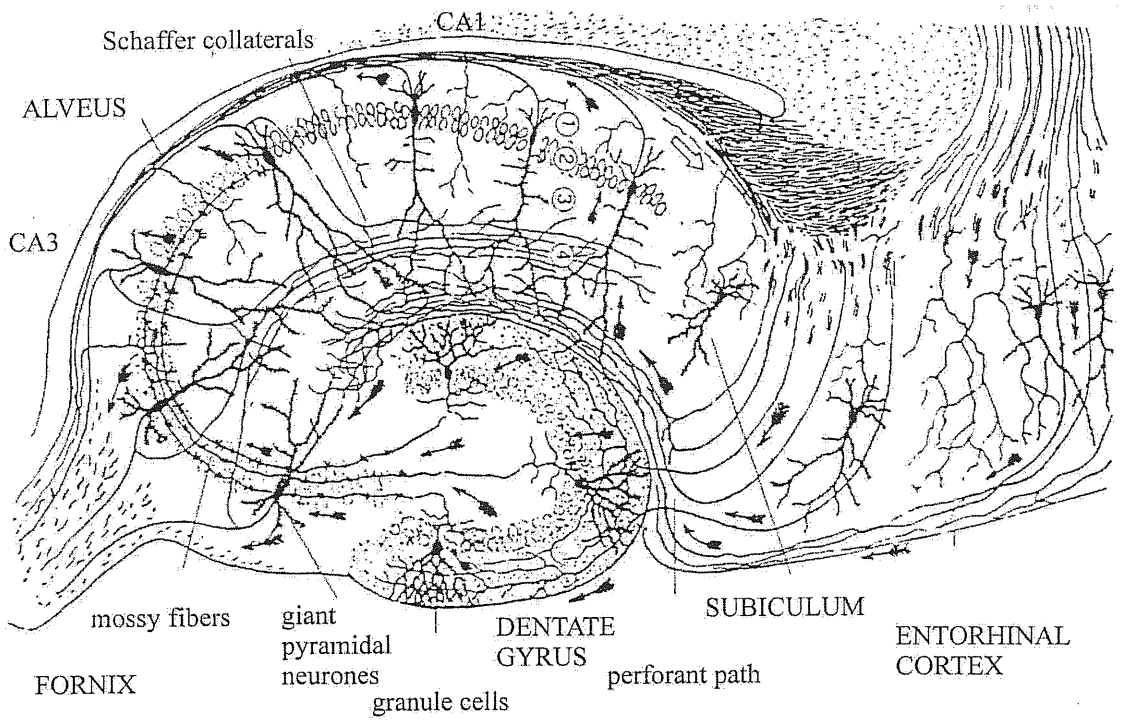

Fig. 5. The organization of the rat hippocampus. Neuronal elements of the hippocampal formation. (1-stratum oriens; 2-stratum pyramidale; 3-stratum radiatum; 4-stratum launosum-moleculare; modified from Cajal, 1911 by Brown and Zador, 1990)

The hippocampal principal inputs arise from:

1) the enthorhinal cortex (EC), which is a main sensory input, provided via the perforant pathway;
2) the septum and diagonal band of Broca complex, containing both cholinergic and GABAergic fibres. They enter the hippocampus via fimbria/fornix, supracallosal striae and the amygdaloid complex;
3) the controlateral hippocampus, via the comissural pathway;

Among other inputs there are noradrenergic projections from locus coeruleus, serotoninergic and non-serotoninergic fibres from the raphe nuclei, dopaminergic projections from the ventral tegmental area, histaminergic projections from tuberomammilary nucleus, vasopressin-containing afferents from the amigdala and not well defined projections from the thalamus (Brown and Zador, 1990; Freund and Buzsaki, 1996).

Granule cells, the principal cells of dentate gyrus, that form the first relay of the so called intrahippocampal trisynaptic excitatory circuit (Andersen et *al.*, 1971; see also Fig. 6 from Freund and Buzsaki, 1996), receive afferents from the lateral EC. The axons of the granule cells are termed mossy fibres. They enter the hilus giving rise to recurrent collaterals back to the dentate gyrus and leave the hilus to form giant en passant boutons, called mossy terminals, on CA3 pyramidal cells.

The pyramidal cells of the CA3 region represent the second stage of the trisynaptic loop. Their most prominent features are large size and complex dendritic spines, called "thorny excrescences".

The last stage of the intrahippocampal loop is represented by the pyramidal cells of the CA1 region which are the target of CA3 pyramidal cell axons termed Schaffer collaterals. The neuronal elements of the trisynaptic loop are the principal hippocampal cells whose bodies form a special layer, *stratum pyramidale (StP)*, see Fig. 7 from Freund and Buzsaki, 1996. The basal dendrites of pyramidal cells arborised in the *stratum oriens (SO)*, whereas apical, radially oriented dendrites spread to the *strata radiatum (SR)* and *lacunosum moleculare (SML).*

Non principal cells (interneurons) represent the majority of the hippocampal cell population and could be found throughout all hippocampal layers. They are diverse in their morphology, neurotransmitter content and function (Freund and Buzsaki, 1996). Since the present study was carried out on the cells from CA1 area of hippocampus, it is worth to concentrate on the structure of this particular region.

1.2.2. CA1 hippocampal region

a) Main connections

In the CA1 field pyramidal cells give rise to a single apical dendrite, whose processes branch out in the *SR* and *SLM,* and to many basal dendrites, that arborize in the *SO.* The pyramidal axon originates from the soma in the region adjacent to the apical dendrite, and only rarely it is seen at the basal dendrite side (Freund and Buzsaki, 1996).

The main excitatory input to the principal cells comes through the Schaffer collaterals and the commissural fibres, that contain axonal branches from ipsilateral and controlateral CA3 pyramidal neurones. These projections terminate onto apical and basal pyramidal dendrites in *SR* and *SO.*

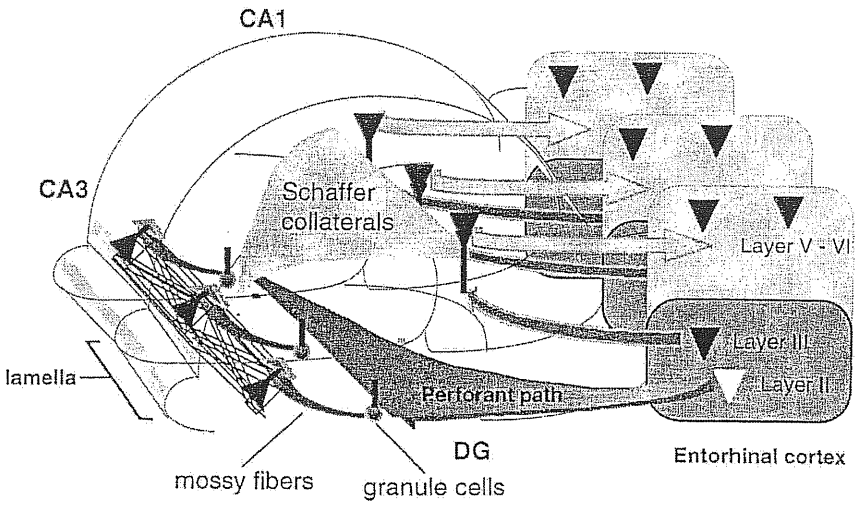

Fig. 6. Schematic representation of the main excitatory connections in the hippocampus proper (trisynaptic circuit) with major hippocampal inputs and projections. (from Freund and Buzsaki, 1996)

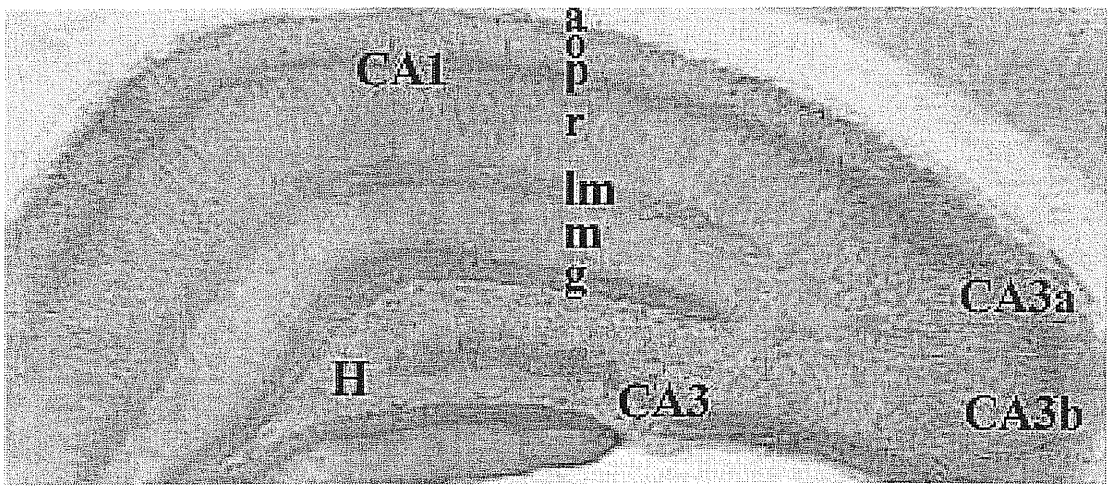

Fig. 7. Coronal section through the dorsal rat hippocampus. CA1,3a-b -regions the CA; o -stratum oriens; p -stratum pyramidale; r-stratum radiatum; lm -stratum lacunosum-moleculare; m -dentate moleculare layer (stratum moleculare); g -granule cell layer (stratum granulosum); H-hilus (modified from Freund and Buzsaki, 1996).

Another important excitatory input is from the EC (Jones, 1993). It makes synapses on the pyramidal dendrites distributed in *SLM*. In the *SO* of the CA1 region there is evidence of local collateral excitatory connections among pyramidal cells (Bernard and Wheal, 1994). However, in the CA1 region they are relatively sparse, with respect to those in the CA3 region, and in the slice preparation the probability of preserving of such connections becomes very low (Bernard and Wheal, 1994). All above mentioned excitatory afferent pathways use glutamate as a transmitter. The excitatory input that comes to the CA1 pyramidal cells through the septohippocampal pathway is however different. Eighty percent of it appears to be made up by cholinergic fibres which establish synapses on the soma and dendrites of the principal cells as well as on the interneurones in all layers of the hippocampus proper (Frotscher and Leranth, 1985; Freund and Buzsaki, 1996). The GABA-ergic afferents representing twenty percent of this pathway, although releasing an inhibitory transmitter, actually produce an overall excitatory effect, due to their inhibition of inhibitory interneurones which leads to pyramidal disinhibition (Freund and Buzsaki, 1996; Toth *et al.*, 1997).

The direct inhibitory input to CA1 pyramidal cells is a local one and comes from the vast network of interneurones. On the basis their morphology (including axonal and dendrite arborization) afferent-efferent connections, and function interneurones can be divided into four classes: axo-axonic, basket, dendritic inhibitory and interneuron-selective cells (Freund and Buzsaki, 1996; see also Fig.8).

The distinguishing feature of *axo-axonic (chandelier) cells* is an axon terminal which presents rows of boutons climbing onto the initial axon segment of the pyramidal cells.

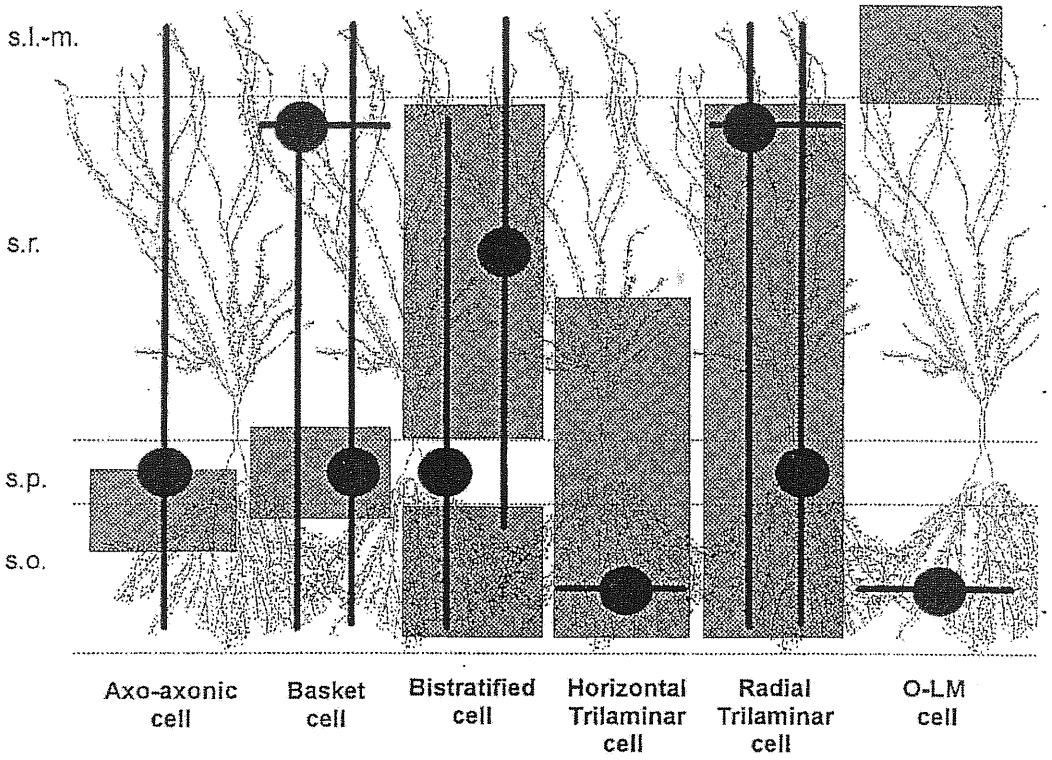

Fig. 8. The scheme of the morphological classification of interneuron types according to dendritic and axonal arborization patterns in the hippocampus. Cell body localization marked as filled circles; thick lines represent predominant orientation and distribution of the dendritic tree; hatched boxes cover the field of axonal arborization of the interneuron; principal cells are shown as shades in the background. (from Freund and Buzsaki, 1996).

The soma of axo-axonic cells is located in the vicinity of *StP* with radial dendrites spreading to all layers.

Basket cells establish heterogenous afferent connections with predominant innervation of the perysomatic region of the pyramidal cells. Their cell bodies are evident in the *SLM* and their dendritic tree spans through *SO*, *SR* to *SML*. The axon arbor is evident in the *StP* and somethimes could reach *SLM*. Both axo-axonic cells and basket cells receive inputs from all major excitatory sources of the hippocampus and thus could be activated in feed-back and feed-forward manner. However, due to their cell body location (*SLM*), basket cells from the CA1 region could be activated predominantly in feed-forward manner, since the recurrent pyramidal terminals in this region are restricted to *SO*.

The third type of interneurones- *dendritic inhibitory cells*- innervates predominantly different segments of the pyramidal dendritic tree and are further divided according to their laminar distribution.

Cells that innervate pyramidal cell dendrites in the *SR* and *SO* are further divided into *bistratified* and *trilaminar*. They have their cell bodies near the *StP* and on the *SO-alveus* border. The radial dendritic tree of trilaminar cells penetrate all layers, while bistratified cells usually do not reach *SLM*. The dendrities of horizontal trilaminar cells ran exclusevly at the *SO-alveus* border. Such a pattern of dendritic arbor enables these cells to receive input from commissural fibres and local recurrent ones and they could be driven in both feed-back and feed-forward manner (horozontal trilaminar cells- mainly in feed-back). Axons of these cells form dense arborization above and below the *StP* , occupying *SO* and proximal parts of *SR*.

The next subgroup of interneurones comprises cells whose axons, cell bodies and dendrities are located in *SR*. These cells establish synaptic contacts on both pyramidal cells and interneurones. In the CA1 region they are exclusively driven by Schaffer collaterals in a feed-forward manner.

Interneurones with their cell bodies in *SLM* have a dendritic and axonal tree predominantly oriented horizontally. However, some of the branches of these cells can travel through *SR* and to the *stratum moleculare* of dentate gyrus and often outside the slice. The other subgroup consists of interneurones projecting across subfield boundaries, so called "backpropagation" neurones (Sik et *al.*, 1994). They are typically visualised in the *SO* of the CA1 region and have a dendritic tree mainly restricted to this layer. Their axons travel to the *SO* and *SR* of the CA3 region, to the hilus, dentate gyrus and even out of the hippocampus via fornix. They are likely to be driven by local collaterals of the CA1 pyramidal cells in a feed-back manner. However, their functional existence in the slice preparation has not yet been proved.

Most, if not all interneurones in the hippocampus are GABA-ergic (reviewed by Freund and Buzsaki, 1996). The majority of spontaneous inhibitory activity recorded *in vitro* from pyramidal cells is thought to be of perisomatic origin (Miles et *al.*, 1996) and thus is due to the activation of basket or axo-axonic cells.

b) Principal neurotransmitters

Glutamate

As mentioned in the previous section, excitatory synaptic events in the CA1 region are mainly mediated by glutamate, which is released by pyramidal cells and EC afferent

fibres. Glutamate, a main excitatory transmitter in the whole vertebrate CNS (Watkins and Evans, 1981), operates via ionotropic and metabotropic receptors.

Ionotropic (ligand-gated) receptors contain an integral ion channel and are divided with respect to their agonist selectivity, structure and electrophysiological properties into two main classes: *N*-methyl-*D*-aspartate (NMDA) and non-NMDA receptors (reviewed by Watkins and Evans, 1981; Scatton, 1993; Hollmann and Heinemann, 1994).

The main characteristics of the NMDA receptor is its permeability to cations, such as Na^+, K^+ and Ca^{2+}, relatively large conductance (40-50 pS), use of glycine as a co-agonist (Johnson and Ascher, 1987) and Mg^{2+} voltage-dependent block, that is relieved upon depolarization (Mayer et *al.*, 1984; Ascher et *al.*, 1988). The latter together with rather slow ion channel kinetics (rise time to peak ~20 ms and bi-exponetial decay with time constants around 40 and 200 ms) gives a high probability of synaptic summation of excitatory events in CA1, thus maintaining synchronization of cell population and participating in the synaptic plasticity (Kauer et *al.*, 1988; Edmonds et *al*, 1995). These receptors are blocked by the selective antagonists *D*-2-amino-5-phosphonopentanoic acid (APV) and (±)3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) or by the channel blocker MK 801.

Non-NMDA receptors are classified into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. In comparison with NMDA receptors, they activate with faster kinetics (rise time of the current is less than 1 ms, decay time constant is between 0.2 and 8 ms), have smaller conductance (about 4-20 pS) and the majority of them is less permeable to Ca^{2+} (Ascher and Nowak, 1988; Iino et *al.*, 1990; Edmonds et *al.*, 1995). One of the characteristic features of these receptors is their fast desensitisation

(Trussell and Fischbach, 1989). The present classification of non-NMDA receptors is based on their pharmacological properties and homology of cloned subunits for the receptor. GluR1-4 subunits denote the AMPA sensitive receptor family, GluR5-7 and KA1-2 subunits identify respectively low- and high-affinity kainate receptor subclasses (reviewed by Hollmann and Heinemann, 1994). Kainate acts potently on the kainate receptors and modestly on AMPA ones, whereas AMPA produce little response on kainate receptors and is highly potent on AMPA receptors. Both AMPA and kainate receptors are blocked by quinoxalinedione antagonists (for example, by 6-cyano-7-nitroquinoxaline-2,3-dione, CNQX, or 6,7-dinitriquinoxaline-2,3(1H,4H)-dione, DNQX); more recently, a selective AMPA antagonist, GYKI 52466, has become available (Paternain et *al*, 1995).

Activation of postsynaptic glutamate receptors produce a depolarizing excitatory response mediated by cationic influx. During low frequency synaptic transmission excitatory postsynaptic potentials in the CA1 hippocampal region are mediated mainly by AMPA and, to a less extend, by NMDA (Watkins and Evans, 1981). However, evidence has accumulated that glutamatergic signaling sometimes can occur purely via NMDA receptors (reviewed by Kullmann and Asztely, 1998).

Metabotropic glutamate receptors (mGluRs) are G protein coupled receptors that are classified according to their amino acid sequence homology, transduction mechanisms and pharmacology into three main subgroups termed as group I (mGluR1, mGluR5), group II (mGluR2, mGluR3), and group III (mGluR4, mGluR6). Group I is potentially activated by quisqualate and 1,3–dicarboxylic acid (ACPD), antagonised by (S)-4-carboxyphenyl-glycine and is coupled to activation of phospholipase C (Aramori and

Nakanishi, 1992; Ozawa et al., 1998). Group II and III are negatively coupled with the adenylate cyclase and potently activated by (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine or ACPD and L-2-amino-4-phosphonobutyrate, respectively (Nakajima et al., 1993). (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine is proposed as a specific antagonist for group II, and α–methyl-L-AP4-as a selective antagonist of group III (Knopfel et al., 1995).

In general, mGluRs can mediate pre or postsynaptic changes in neuronal excitability. In particular, at postsynaptic level activation of mGluRs produces a slow depolarization with an increase in firing rate. This excitatory effect is attributed to reduction in several K^+ conductances, such as I_{leak}, I_M, I_{AHP} (Charpak et al., 1990, Ozawa et al., 1998) and an increase in Ca^{2+} activated non-specific cationic conductances (Crepel et al., 1994). Stimulation of mossy fibers and bursting in CA1 presynaptic neurones disclose an mGluR mediated EPSP, that has a very slow time course (rise time 5 s, duration 60 s; Charpak and Gahwiler, 1991; Bianchi and Wong, 1995).

At presynaptic level activation of mGluRs is shown to down-modulate both excitatory and inhibitory transmission. This effect is suggested to be mediated by inhibition of presynaptic L- and N-type Ca^{2+} channels (Kobayashi et al., 1996). Activation of the mGluR2 was shown to be involved in the induction of LTD at the mossy fiber synapse (Kobayashi et al., 1996).

NMDA, non-NMDA and mGlu receptor binding sites are dense in the CA1 region and mRNAs of the receptor subunits are also expressed in this area (for review see Ozawa et al., 1998).

Modulation of the glutamatergic response on the pyramidal cells could be achieved in two ways, presynaptically and postsynaptically. Presynaptic mechanisms include biosynthesis of L-glutamate, its release and degradation or mGluR autoreceptor-mediated negative feedback (Swartz and *al.*, 1993). At postsynaptic level non-NMDA events can be limited by rapid desensitization which is process inhibited by cyclothiazide (for AMPA receptors, Vyklicky et *al.*, 1991) or by concanavalin A (for kainate receptors, Partin et *al.*, 1993). Functional upregulation of native non-NMDA receptors involves cAMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase, although the sites for phosphorylation and the functional consequences of this phenomenon remain presently unclear (reviewed by Smart, 1997). Modulation of NMDA activity can be brought about through various receptor sites (Scatton, 1993), like for example, upregulation by polyamines (Benveniste and Mayer, 1993), or block by Mg^{2+} in voltage-dependent manner (Mayer et *al.*, 1984; Ascher et *al.*, 1988) or by Zn^{2+} (Scatton, 1993). Direct activation by protein kinase C of certain phosphorylation sites is known to potentiate NMDA response (reviewed by Smart, 1997).

GABA

γ-Amino-butyric acid (GABA) is the main inhibitory transmitter in vertebrate CNS. In the CA1 region it is released from interneurones and binds to specific receptors termed $GABA_A$ and $GABA_B$.

The $GABA_A$ receptor is a ligand-gated channel permeable to Cl^- and blocked by bicuculline or picrotoxin in a competitive or non-competitive manner, respectively

(Sivilotti and Nistri, 1991). The $GABA_A$ receptor mediates fast inhibitory postsynaptic potentials.

The $GABA_B$ receptor is coupled via G-protein and intracellular second messengers to cation channels (K^+,Ca^{2+}), and is blocked by phaclofen or 2-hydroxy-saclofen. It is responsible for the late component of IPSP and modulates synaptic transmission by presynaptic inhibition of transmitter release (Dutar and Nicoll, 1988; reviewed by Bowery, 1993).

GABAergic markers (glutamic acid decarboxylase, GABA-transaminase, GABA itself) are present throughout the whole hippocampus (Sivilotti and Nistri, 1991). Positive staining is especially dense around the soma and dendritic fields of pyramidal cells in the CA1-CA3 regions and also evident in the dendritic layers of GABAergic interneurones (Woodson et *al.*, 1989). Most of the GABAergic terminals in the CA1 area are intrinsic, but there is an afferent input from septum, whose symmetric synapses are evident in the *SO*.

Interneurones have been shown to express high levels of $GABA_A$ receptors (review by Freund and Buszaki, 1996). A comparison of the $GABA_B$ receptor mRNA distribution and receptor binding sites revealed a discrepancy in some brain regions, including hippocampus, where receptor transcripts are observed in the *StP* of the hippocampus proper, whereas receptor binding sites were reported in the molecular layers (Kaupmann et *al.*, 1997). This fact could be tentatively explained assuming that the cloned receptors represent a low-affinity $GABA_B$ receptor.

Activation of $GABA_A$ receptors can produce two opposite postsynaptic effects in hippocampus, namely hyperpolarization and depolarization. While it was first thought

that distinct receptor subpopulations localized on the soma and dendrites were responsible for this different action, more recent data go against this theory (Gaiarsa et *al*, 1995). A possible explanation for this phenomenon relies on the amount of GABA released from presynaptic terminals whereby a small amount of transmitter produces hyperpolarization (mediated by Cl^- influx), while a large release evokes depolarization (or a biphasic response) due to efflux of bicarbonate and Cl^- and reduction in the Cl^- electrochemical gradient (Grover et *al.*, 1993; Staley et *al.*, 1995).

It has been shown that in the CA1 region $GABA_B$ mediated postsynaptic responses, consisting of late membrane hyperpolarisation mediated by K^+ efflux, require higher intensity of stimulation than the ones elicited by $GABA_A$ and that spontaneous release of GABA apparently fails to activate the $GABA_B$ system (Collingridge et *al.*, 1984; Dultar and Nicoll, 1988; Otis and Mody, 1992). This phenomenon could be attributed to differential accessibility of the receptors in the postsynaptic area as well as to different receptor affinities. The readily accessible, high affinity $GABA_A$ receptors require less transmitter for their activation than $GABA_B$ receptors (Chu et *al.*, 1990; Mody et *al.*, 1994). The slow time course of $GABA_B$ mediated responses also reflects electrotonic filtering of the signal by the dendrites (Spruston et *al.*, 1994) as, according to Newberry and Nicoll (1985), $GABA_B$ receptors are preferentially located on pyramidal cell dendrites whereas $GABA_A$ ones are on the soma.

At rest pyramidal cells are under tonic inhibition by interneurones. Modulation of this GABAergic input to pyramidal neurones can be achieved by 1) changing presynaptic transmitter release, a mechanism used for example by opiates (Capogna et *al.*, 1993) 2) by changing transmitter uptake (Thompson and Ghawiler, 1992), 3) by changing

postsynaptic receptor activity with drugs such as barbiturates, benzodiazepines, volatile anesthetics which bind to receptors modifying their kinetics (Otis and Mody, 1992) or with protein kinases (like cAMP-dependent proteinkinase or protein kinase C) interacting with the receptor at the phosphorylation site (Smart, 1997).

Acetylcholine

The hippocampus receives a large cholinergic input via the septohippocampal pathway from the medial septal nucleus and the diagonal band of Broca (Frotscher and Leranth, 1985; Dutar et *al.*, 1995; Freund and Buzsaki, 1996). In the CA1 region cholinergic fibers innervate both principal cells, forming symmetric synaptic contacts on their bodies and dendrites, and non-principal cells, establishing asymmetric contacts on their dendritic arborizations (Frotscher and Leranth, 1985). A small number of cholinergic non-pyramidal cells, that show morphological characteristics of GABAergic interneurones, has been observed intrinsic to the hippocampus (Freund and Buzsaki, 1996). They are mainly found in the *SLM* and are sparse in *SR*, *StP* of the CA1 area and rarely present in the CA3 region.

Although the majority of immunohistochemical studies on cholinergic innervation has been performed in rats, the pattern of cholinergic innervation in mouse hippocampus is quite similar to the one seen in rats (Frotscher and Leranth, 1985; Kitt et *al.*, 1994).

In the hippocampus both muscarinic and nicotinic cholinergic receptors are present. Here only the muscarinic part of the hippocampal cholinergic system will be considered.

Up to date five distinct muscarinic receptors (M1-M5) have been cloned and expressed in neuronal tissue (Bonner et *al.*, 1988; Waelbroeck et *al.*, 1990). They belong to the metabotropic receptor family, differ in pharmacology, distribution, and electrophysiological properties and are coupled to diverse second messenger systems. Carbachol is a broad spectrum muscarinic agonist, that activates all types of muscarinic receptors, but is also effective on nicotinic ones. Atropine is used as a broad spectrum antagonist of muscarinic responses. Many compounds have been generated to identify subtypes of muscarinic receptors, but at present neither selective agonists nor antagonists are readily available to discriminate receptor subtypes very sharply.

The lack of very selective ligands poses problems for the precise labelling of receptor binding sites. In the hippocampus a first detailed study of receptor distribution has been done by Levey et *al.* (1995) using subtype specific antibodies against muscarinic acethylcholine (ACh) receptor proteins. The localisation of receptor proteins, designated as m1-m5, has turned out to be in closer agreement with their receptor mRNA signals, than with the binding sites (M1-M5), and revealed a distinct laminar distribution (Vilaro et *al.*, 1993; Levey at *al.*, 1995 and references therein). Combining results from immunohistochemical studies and lesion experiments allows to consider M2 and M4 receptors as pure presynaptic, when M1, M3 (and perhaps M5) receptors as postsynaptic.

In the hippocampus postsynaptic muscarinic effects are due to M1 and M3 receptor subtype activation, while presynaptic ones are ascribed to M2 and M4 receptors subtypes (McKinney, 1993). The most prominent postsynaptic effect of ACh is a slow depolarisation associated with enhancement of cell excitability (Cole and Nicoll, 1983;

Muller and Misgeld, 1986). Decrease in several K^+ conductances underlies this phenomenon, contributing also to facilitation of repetitive firing, depression of the afterhyperpolarization (AHP) and spike modulation (Krnjevic et *al.*, 1971; Bernardo and Prince, 1982; Cole and Nicoll, 1984). The decrease in K^+ conductance involves block of ionic currents such as I_M (Brown and Adams, 1980;), I_{AHP} (Cole and Nicoll, 1984), I_{LEAK} (Madison et *al.*, 1987; Benson et *al.*, 1988) and I_A (Nakajima et al., 1986). ACh acting on muscarinic receptors is also known to potentiate voltage-activated potassium currents (Zhang et *al.*, 1992; Wakamori et *al.*, 1993) and to reduce sodium conductance (Cantrell et *al.*, 1996), that could be responsible for the more rarely observed hyperpolarizing effect. There is also evidence that ACh inhibits voltage dependent Ca^{2+} channels (Toselli et *al.*, 1989). Muscarinic receptors enhance the NMDA responses postsynaptically after an initial depressant effect (Markham and Segal, 1990).

Presynaptically ACh produces inhibition of transmitter release, including its own release (Valentino and Dingledine, 1981; Raiteri et *al*, 1984). The most important effect to this extent is cholinergic disinhibition that consists in decrease of tonic GABAergic drive on pyramidal cells evident as decreased IPSP amplitude and an induction of multiple spikes (Ben-Ari et *al.*, 1981; Haas, 1982). The mechanism of this presynaptic effect is not completely clear: a reduction in Ca^{2+} conductance and/or an increase in K^+ conductance can account for it (McKinney, 1993).

All muscarinic responses are G-protein coupled and mediated by second messenger cascades (reviewed by McKinney). Activation of M1, M3 and M5 receptors is thought to involve the phosphonositide cycle (inositol triphosphate and diacylglycerol formation,

Ca^{2+} mobilisation from intracellular stores and modulation of cationic fluxes). This pathway is not sensitive to pertussis toxin (PTX). M2 and M4 are linked (via a PTX-sensitive mechanism) to inhibition of adenylate cyclase with a consequent shunting of voltage-gated Ca^{2+} currents (for review see McKinney, 1993).

The role of ACh in the CNS is mainly due to activation of muscarinic receptors and has a more neuromodulatory than transmitter function (for review see Jerusalinsky et al., 1997). In the hippocampus ACh is involved in the modulation of synaptic plasticity (Bliss and Lomo, 1973; Martinez and Derrick, 1996) and formation of theta rhythm (Krnjevic, 1993; Freund and Buzsaki, 1996).

Since the cholinergic system is suggested to be involved in memory processing, the question of modulation of its synaptic transmission is of special interest from a functional point of view. To date few studies have addressed this issue at cellular level in the hippocampus (Maura et *al.*, 1989; Dultar et *al.*, 1989; Morton and Davies, 1997), albeit much work is done using microdialysis and autoradiography (reviewed by Decker and McGaugh, 1991). The most common locus for cholinergic transmission regulation is presynaptic. For example, tonic inhibitory control of ACh by serotonin (Maura et al., 1989), galanin (Dultar et al., 1989) or adenosine (Morton and Davies, 1997) has been reported. The postsynaptic level of cholinergic modulation mainly implies interaction with transduction mechanisms activated by muscarinic receptors (phosphorylation of G-protein, inhibition of adenylate cyclase and activation of phospholipase C).

1.3. SP IN HIPPOCAMPUS

According to immunohistochemical studies done by Iritani et al. (1989) and by the group of Leranth (Seress and Leranth, 1996; Borhegyi and Leranth, 1997), SP is present in the mammalian hippocampus. SP-positive cell bodies are mainly in the *SO* of the CA1-3. Fewer numbers are evident in *SR* and *StP* of the Ammon's horn, in the dentate gyrus and in stratum granulosum of the hilus (Davis and Kohler, 1985; Borhegyi and Leranth, 1997). The localisation, dendritic tree and axon projections pattern of these neurones in the hippocampus proper resembles that of the bistratified interneurones (Borhegyi and Leranth, 1997).

The SP-immunoreactive fibres of the hippocampus are divided into two groups: those of intrinsic origin with axons establishing symmetric synapses, and those of extrinsic origin that form asymmetric contacts on the postsynaptic cells (Seress and Leranth, 1996; Borhegyi and Leranth, 1997). SP projections to the hippocampus arrive from the supramammillary area (Gall and Selawski, 1984; Leranth and Nitsch, 1994) and the posterior hypothalamus (Yanagihara and Niimi, 1989). Their target cells are observed in *StP* and *SR* of CA2-CA3a area as confirmed by retrograde labelling and fornix-fimbria transection studies (Borhegyi and Leranth, 1997). A dense intrinsic SP fibre network is found in *SO* and *SR* of the CA1 region, whereas it is less developed in the CA3 region (Borhegyi and Leranth, 1997). In rat dentate gyrus SP fibres are very sparse (Davis and Kohler, 1985; Borhegyi and Leranth, 1997) in comparison for example with monkey, guinea pig or man in whom this region is abundantly supplied with SP (Seress and Leranth, 1996; Iritani et *al.*, 1989; and Del Fiacco et *al.*, 1987).

Both extra-hippocampal and intrinsic SP-containing fibres were once supposed to terminate mainly on dendrites and somata of principal cells, creating a discrepancy between the distribution of NK receptor immunoreactivity (restricted to interneurones) and SP-containing axons (Acsady et *al.*, 1997). However, recent electron microscopic analysis of the rat hippocampus has also shown non-pyramidal cells among potential targets for SP containing fibres (Borhegyi and Leranth, 1997). Nevertheless, the mentioned mismatch, typical of peptide neurotransmission, has been noticed in rat hilus and dentate gyrus, where the most robust SP receptor labelling contrasted with the poor endogenous SP supply (Nakaya et *al.*, 1994; Acsady et *al.*, 1997; Borhegyi and Leranth, 1997).

In the hippocampus SP receptors are localised only on interneurones (Nakaya et *al.*, 1994), especially GABAergic ones (Acsady et *al.*, 1997), that are distributed throughout all hippocampal layers. NK receptors are considered to belong mainly to the NK_1 receptor type (Humpel and Saria, 1993). The mRNA for the NK_1 receptor is shown to be present in non-pyramidal layers of the hippocampus as well (Maeno et *al.*, 1993).

In spite of wide distribution of SP with its binding sites in hippocampus and a detailed characteristic of this network from the anatomical point of view, the function of SP in the hippocampus remains uncertain. The peptide has been suggested to facilitate learning in the rat (Huston and Hasenohrl, 1995; Sprick et *al.*, 1996). The work by Taoka and co-workers (1996) discovered an enhanced NK_1 receptor mRNA level during the first days of life implying for SP a role in plastic changes during early hippocampal development. There is evidence that SP could play a role in seizure activity. Sperk et *al.* (1990) have reported initial decrease in SP as well as in total NKA and NKB immunoreactivities with

subsequent long-lasting substantial increase after acute kainate-induced sezures in the hippocampus. More recent data from the same laboratory have shown that these changes are restricted to NKB tachykinin subclass, evident in the granule cells and interneurones of DG and in the pyramidal layer of the CA3 region (Marksteiner et *al.*, 1992). However, the studies of Zachrisson et *al.* (1998) using the *in situ* hybridization method, showed an increase in the levels of preprotachykinin A mRNA in the CA1 region one hour after kainate injection. The same group has also demonstrated that the pretreatment with the selective NK1 receptor antagonist CP-122,721-1 produces seizure inhibiting and neuroprotective effects on the CA1 hippocampal neurones following kainate administration. On hippocampal cultures Yankner et al. (1990) have shown that the neurotoxic effects of β-amiloid protein can be mimicked by application of SP antagonists and reversed by specific NK agonists, suggestive of a potential SP involvement in some mechanisms associated with Alzheimer's disease.

Regarding the function of SP in hippocampus, there are few electrophysiological studies examining the mechanism of action of SP at a single cell level. For instance, the intracellular study by Dodd and Kelly (1981) has found no direct effect of SP on pyramidal cells of the CA1 region. On the other hand, Dreifuss and Raggenbass (1986) using extracellular recordings from the same brain region have demonstrated an excitatory action of the peptide on presumably non-pyramidal cells. Recently Lieberman and Mody (1998) employing patch clamp cell-attached recordings in acutely dissociated granule cells from the DG discovered a direct enhancement of single NMDA channel function by SP and its selective NK1 receptor agonist. Thus, the locus and mode of action of SP in the hippocampus remain obscure.

II. AIM OF THE PROJECT

The aim of the present project was to investigate the action of SP in the CNS using as a model the CA1 region of the hippocampus. In particular, this study addressed the following questions:

a), could SP exert any direct effect on CA1 pyramidal cells, changing intrinsic membrane properties, and thus playing a neurotransmitter role;

b), could SP (like many other neuropeptides) possess a modulatory activity in the hippocampus by affecting existing neurotransmitter systems (glutamatergic, GABAergic, cholinergic);

c), what type of NK receptors was involved;

For this purpose electrophysiological experiments were performed on the mouse hippocampal slice preparation. By choosing this animal species it was planned to obtain a database of the electrophysiological actions of substance P which may then be used as reference for future studies on transgenic mice in which discrete changes in SP availability or targets would be genetically induced. In the present project field potential and membrane potential changes were recorded either extracellularly or intracellularly in current clamp conditions using sharp microelectrodes. The latter approach has been chosen to preserve the intracellular milieu of the recorded cell (see for instance Levitan and Kramer, 1990) which is important to detect any direct action of SP, known to be mediated by intracellular second messengers via G-protein coupled receptors (for reviews see Otsuka and Yoshioka, 1993; Regoli *et al.*, 1994).

III. METHODS

3.1. Slice preparation

For the experiments CD-1 mice of both sexes (1 to 2 month old) were used. They were housed at room temperature (20-22°C) with unrestricted access to water and pellet food. Animals were anesthetized with diethylether and decapitated. The brain was removed from the skull and placed into a cold (2-4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing (mM): NaCl 124, KCl 3.7, $MgSO_4 7H_2O$ 2, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10. The two hippocampi were carefully dissected free, laid on an Agar bed (4 gr Agar in 100 ml 0.9% NaCl) and cut transversely into 400-500 μm thick slices using a McIlwain tissue chopper. Slices were allowed to recover for at least one hour in the same ACSF equilibrated with 95% O_2 and 5% CO_2 to Ph 7.3-7.4 at room temperature, before a single slice was transferred to a submerged recording chamber. In the chamber the slice was laid on the nylon mesh and continuously superfused (2.5-3 ml/min) with ACSF at 30-33 °C.

3.2. Intracellular recordings

Intracellular recordings from hippocampal CA1 neurons were obtained using an Axoclamp 2A amplifier (Axon Inst., Foster City, CA) under discontinuous current clamp conditions (sampling rate approximately 3 kHz) or in conventional bridge mode when balance was regularly checked during the experiment.

Microelectrodes were prepared from borosilicate glass tubing of 1.2 or 1.5 mm o.d. (Clark Electromedical Instruments) and filled with either 3 M KCl (40-60 MΩ) or 4 M potassium acetate (60-100 MΩ).

The electrode was positioned in the pyramidal cell layer of CA 1 region under visual control. After that, while advancing through the slice with the help of a Narishige hydraulic micromanipulator, short hyperpolarising pulses at a high frequency (10ms, 0.3-04 nA, 10 Hz) were applied. A sudden increase in resistance was indicative of the approaching the cell surface. At this particular moment an impalement was obtained with the aid of a large transient current, generated by the amplifier (clear switch operation). Following the impalement, if necessary, cell was stabilized by continuous injection of negative current (not more than -0.5 nA).

Cells were considered to be pyramidal neurones in accordance with the electrophysiological criteria proposed by Lacaille (1991), that included typical spike and accommodation characteristics in response to single depolarising current pulses (0.3 to 1.0 nA, 50 or 500 ms duration). To monitor the cell input resistance continuously, hyperpolarizing current pulses (-0.05 to -0.1 nA range, 500 ms duration) at a frequency of 0.1 Hz were routinely delivered. Afterhyperpolarization (AHP), spike current rheobase, latency of the first spike and spike accommodation were evaluated using depolarizing current steps (0.1-0.6 nA; 500-700 ms duration). When membrane potential was changed (due to the effect produced by a substance), cell was manually "clamped" at the membrane potential equal to the one in control by continuous current injection. In order to generate I/V curves current pulses (500 ms) of both polarities in the range from -0.6 to +0.6 nA were applied.

To elicit excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs), focal electrical stimulation with a pair of twisted NiCr electrodes placed in the stratum radiatum of the CA1 region (orthodromic stimulation of the Schaffer collaterals/commissural pathway; 0.1 ms, 0.05 Hz) was employed. The intensity of the stimulation was adjusted to be equal in amplitude ½ of the maximum evoked response. Average of 5-10 responses was calculated.

Slow cholinergic exitatory postsynaptic potentials (sEPSPs) were elicited by a train of 30-50 pulses at 15-30 Hz of 30 μs duration when the twisted NiCr isolated wire was placed at the stratum oriens/alveus border in CA1 region in the continuous presence of edrophonium chloride (3 μM), an acethylcholineesterase inhibitor.

To record $GABA_A$ receptor mediated IPSPs either potassium acetate-filled electrodes or KCl-filled electrodes in the presence of kynurenic acid (1 mM), a broad spectrum antagonist of glutamate receptors, were used.

Spontaneous synaptic events (either in control solution or in the presence of TTX) were measured as previously reported (Atzori and Nistri, 1996). The Axograph program (Axon Instruments Inc., Foster City, CA) on a Power Macintosh computer was used for off-line analysis of the synaptic responses.

The voltage signal was acquired using p-CLAMP software (Axon Instruments Inc.), filtered at 3 kHz, monitored on a digital storage oscilloscope, a chart recorder and stored on a PC hard disc and videotape for further analysis.

3.3. Extracellular recordings

Extracellular recordings were obtained from stratum radiatum of the CA1 hippocampal area with a glass microelectrode filled with 2 M NaCl (resistance 2-5 MΩ).

To evoke field potentials orthodromic stimulation of the Schaffer collaterals/commissural pathway was performed as explained in intracellular section. Extracellular recordings were accepted whenever met the following criteria: field potential amplitude not smaller than 1 mV; the ratio between field potential amplitude and presynaptic volley higher than 3, (presynaptic volley did not change during the experiment); field potential waveform with a smooth shape (Schwartzkroin, 1981). For routine monitoring of synaptic transmission the stimulus intensity was chosen from the stimulus input-output curve and set at a half of the maximal amplitude of the evoked response. Input/output curves were constructed by stepwise changing the stimulus intensity.

In order to dissected out the AMPA mediated glutamatergic component of the response bath application of the NMDA antagonist D-2-amino-5-phosphonopentanoic acid (APV, 20 μM) was used. The NMDA component was pharmacologically isolated with superfusion of the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 4-10 μM); in several experiments 6,7-dinitriquinoxaline-2,3(1H,4H)-dione (DNQX, 3 μM) was also used.

For "mini-slice" experiments recordings were first performed from the entire preparation and then the protocol was repeated after a desired area of the hippocampus was either completely cut off (for CA3 or dentate gyrus) or severed (stratum oriens, stratum radiatum) with the help of a sharp scalpel.

Data were collected every 60 s after averaging three responses (at 20 s interval). The "Long term potentiation" program (version 0.92, kindly provided by W. W. Anderson,

Bristol University, England) was employed for acquisition and on-line analysis of field potential amplitude and slope.

3.4. Drugs and data analysis

All drugs were dissolved in ACSF to the final concentration mentioned in the text. Bath application was via a three-way tap superfusion system at a rate of 2.5-3 ml/min, and the solution was fed by gravity or pumped electrically by a peristaltic pump.

SP, substance P methylester (SPME), tetrodotoxin (TTX), carbachol (CCh), edrofonium chloride, atropine sulfate and bicuculline methiodide were obtained from Sigma. APV, AMPA, NMDA, CNQX and DNQX were purchased from Tocris Cookson. SR 140333 ((*S*)-1-[2[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenyl)-piperidin-3-ethyl]-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride) was generously donated by Dr. X. Emonds-Alt (Sanofi Research).

Data are presented as means ± s.e.m. Statistical analysis was done using Student's *t*-test for parametric data and the one way ANOVA test for non-parametric results.

IV. RESULTS

4.1. SP depressed synaptic transmission in hippocampus via a GABAergic mechanism

We used extracellular recordings as a preliminary approach to investigate the effects that SP elicits in the mouse hippocampus. In Fig. 9A a typical example of such a recording is shown in which after 4 min application SP (2 μM) depressed the amplitude and slope of the field potential by 28% and 20%, respectively. This effect was already evident at the second min of application, reached its maximum at 4 min and waned by 5 min of washout. Re-applying the same concentration immediately (after 5 min wash) produced a strong attenuation of the response or no effect. As a 20-30 min rest from the last application of SP was usually sufficient for the slice to regain its sensitivity to the peptide, such a protocol was adopted for long-term tests. This protocol allowed for up to four applications of the peptide with reproducible and reversible effects.

SP (2-4 μM) uniformly depressed the field potential amplitude through a wide stimulus range, leading to a nearly parallel shift of the input/output curve (see Fig. 9B n=4). As shown in Fig. 9C, the depressant action of SP (2-4 μM) was fully antagonized by the selective NK_1 blocker SR 140333 (4 μM; Emonds-Alt *et al.*, 1993). The peptidase-resistant analogue SPME (0.1 μM) elicited a quantitatively similar effect as SP, that was equally sensitive to SR 140333 antagonism (see hatched bars in Fig. 9C). These data thus suggested that the action of SP was mainly mediated by NK_1 receptors. The fact that twenty times less concentration of SPME mimicked the effect of SP (0.1 μM against 2 μM) indicated that substantial tissue inactivation of SP took place and prompted the use

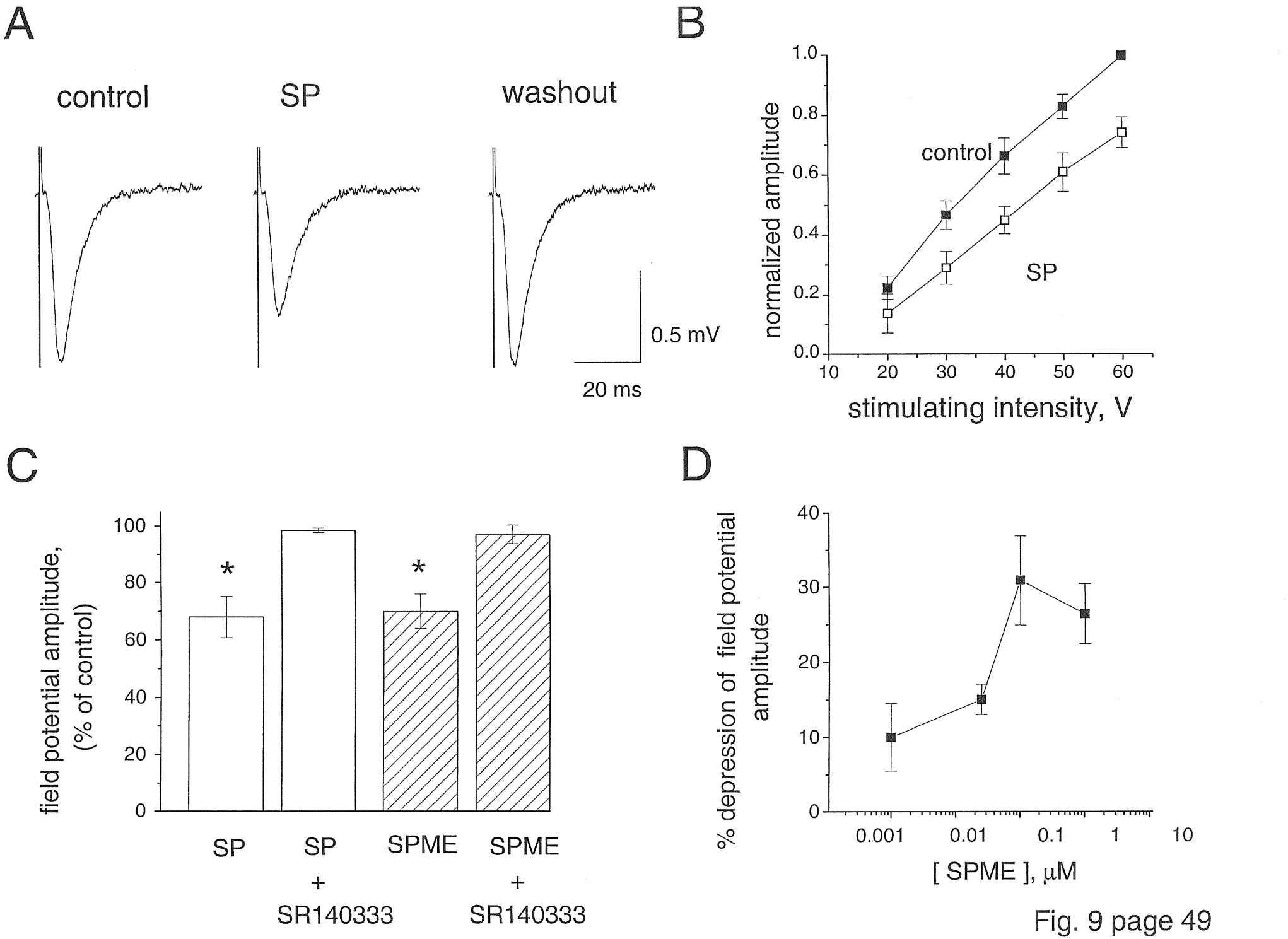
A
control
SP
washout
0.5 mV
20 ms
B
normalized amplitude
control
SP
stimulating intensity, V
C
field potential amplitude, (% of control)
SP
SP + SR140333
SPME
SPME + SR140333
D
% depression of field potential amplitude
[SPME], μM

Fig. 9 page 49

Fig. 9 Effect of SP and SPME on the amplitude of CA1 field potentials. A: field potential in control solution, in the presence of 2 μM SP (4 min application) and after 20 min washout. Note reversible reduction in the amplitude of the response. Traces are averages of 3 responses. B: input/output relation between the amplitude of field potentials and the stimulation intensity in control or in 4 μM SP solution. Note nearly parallel shift of the curve due to equivalent degree of depression through the observed range. Datapoints are from 4 slices. C: histograms of change in the field potential amplitude (expressed as % of control value) obtained first in 2-4 μM SP (open bars) or 0.1 μM SPME (hatched bars) solution and then re-tested in the presence of the selective NK_1 receptor antagonist SR 140333 (4 μM). Note similar degree of depression by SP or SPME, and that the antagonist blocks both effects. Asterisks indicate that data are significantly different from control ($P<0.02$; n=4). D: log concentration-response curve for the depressant action of SPME on field potentials (nò4). Effect appears to saturate at 0.1 μM concentration.

of the stable compound SPME for further experiments. The dose-dependent depression of the field potential amplitude by SPME is demonstrated in Fig. 9D. The maximum reduction was observed with 0.1 μM concentration (31±6 % decrease in response amplitude; n=19). Changes in slope value of the field potential in the presence of SPME were always paralleled by amplitude changes and, on the same sample of slices, were equal to 28±4 %.

Field potentials elicited by *SR* stimulation are known to comprise a glutamatergic response mediated by activation of non-NMDA and NMDA receptor classes (Mayer and Westbrook, 1987). The experimental protocol with SPME application was repeated during pharmacological isolation of each glutamate receptor classes in order to assess if one of them was selectively affected by SPME. In the presence of APV (20 μM) or CNQX (10 μM) SPME (0.1 μM) retained its depressant action (see Fig. 10A), suggesting that AMPA- or NMDA- receptor mediated responses were affected in a similar way. The depressant effect of SPME on the field potential in control solution or on the NMDA and non-NMDA components was however blocked by bicuculline (10μM), an antagonist of $GABA_A$ receptors (Fig. 10B). It should be noted that in the presence of bicuculline the same stimulation intensity used to elicit the field potential in control now evoked a much larger amplitude response which decayed slowly with superimposed oscillations, typical of disinhibited pyramidal cell activity (not shown; Crepel et al., 1997 and references therein). In a view of such changes, in the presence of bicuculline the stimulation intensity was reset to a lower value which evoked a field potential amplitude similar to the one observed in normal Krebs solution.

Fig. 10C demonstrates that on the same slice preparation the reduction in field potential amplitude produced by SPME in control Krebs solution (40% reduction, left panel Fig. 10C) was almost absent in bicuculline solution (12% reduction, right panel). This result suggests that for the depressant action of SPME on hippocampal synaptic transmission intact GABAergic mechanisms were essential.

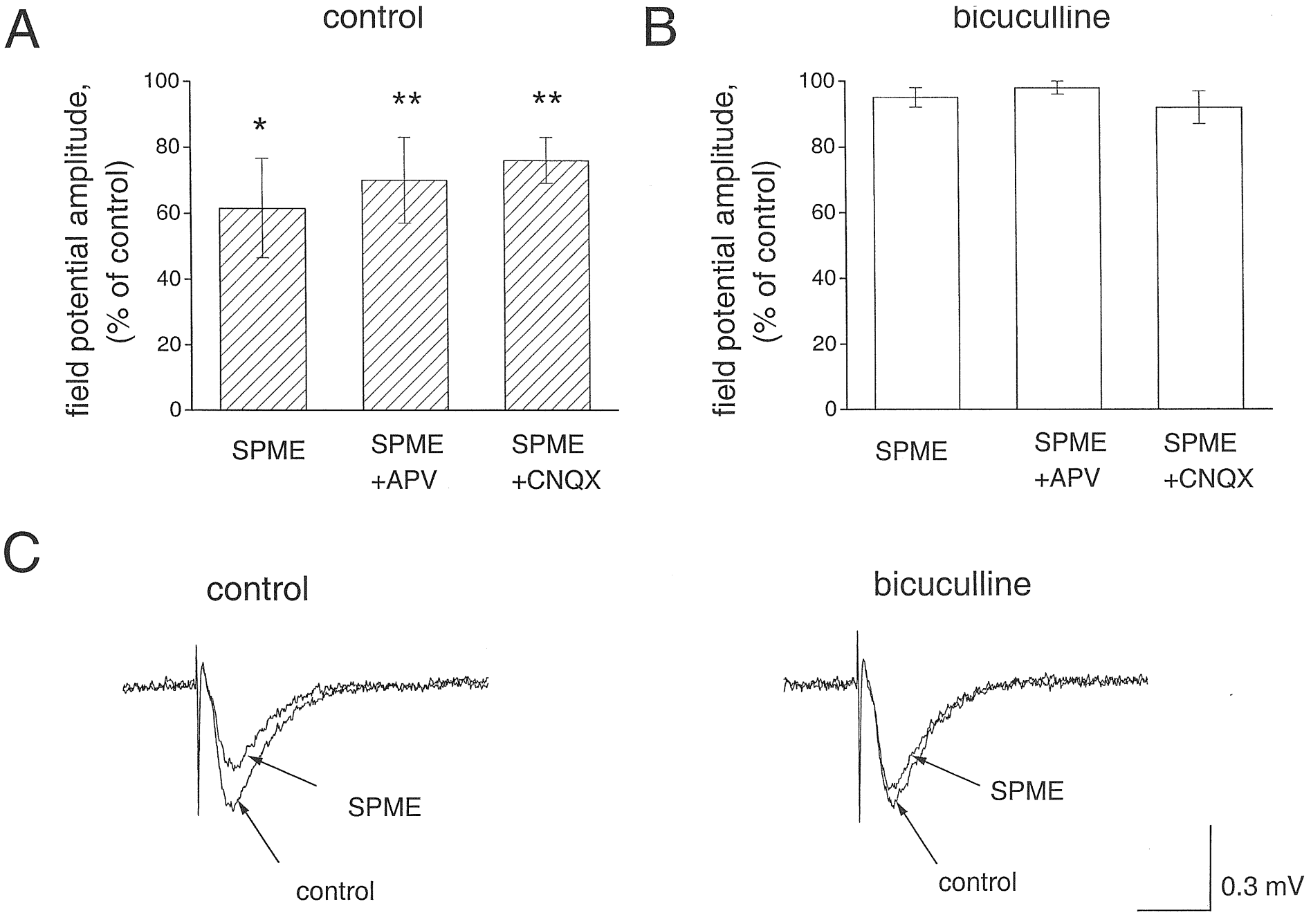

Fig. 10 page 52

Fig. 10 The depressant effect of SPME does not preferentially involve a glutamatergic component of the field potential and is bicuculline sensitive. A: histograms of field potential amplitude in SPME (0.1 μM) alone or following application of APV (20 μM) or CNQX (10 μM). *=P<0.02, **=P<0.05; n =4-9 slices. B: histograms depicting lack of effect of SPME alone or in the presence of APV or CNQX following application of bicuculline (10 μM). Data are from the same slices shown in A. C: representative examples of field potential depression by SPME (0.1 μM) before and after application of bicuculline. Traces are averages of 3 responses.

4.2. Localisation of the effect of SPME

In a separate set of experiments we tried to evaluate the role of different components of the hippocampal circuitry in the depressant action of this neuropeptide. For this purpose, once the effect of SPME on the field potential was observed in an intact preparation (Fig. 11A), a cut was made to separate the dentate gyrus, or the CA3 region, or one of the strata of the CA1 region (*SLM*, *SO*) from the place of recording. The drug application protocol was repeated after a period of time sufficient for a slice to regain stable activity (usually after 20-40 min).

When the cut was made to remove CA3 region (see dashed line in the scheme of Fig. 11B), the amplitude of the field potential recorded in *SR* was decreased by 25±5% with respect to the value obtained in the entire slice (n=14). The depressant action of SPME was still present, but only in 4 slices the degree of this depression was the same as in the intact preparation. In the majority of slices with CA3 ablation (10/14) the depression produced by SPME became much weaker than that recorded from the whole preparation, although remained significantly different (compare trace exemplified in Fig. 11B with the one in Fig. 11A). In SPME solution the amplitude of the field potential was equal to -0.42±0.05 mV versus -0.52±0.05 in control, whereas before the cut it was it was -0.44±0.05 mV versus -0.70±0.06 mV respectively ($P<0.001$ for both). Hence, in the presence of SPME field potential amplitude was depressed only by 19±4% and the slope was reduced by 18±5% against the reduction of 36±5% and 39±6%, respectively, observed before the cut ($P<0.001$ for both results).

When the dentate gyrus was ablated (not shown), in 8/9 slices SPME (0.1 μM) evoked a depression smaller than that produced before in the unlesioned slices (29±8%

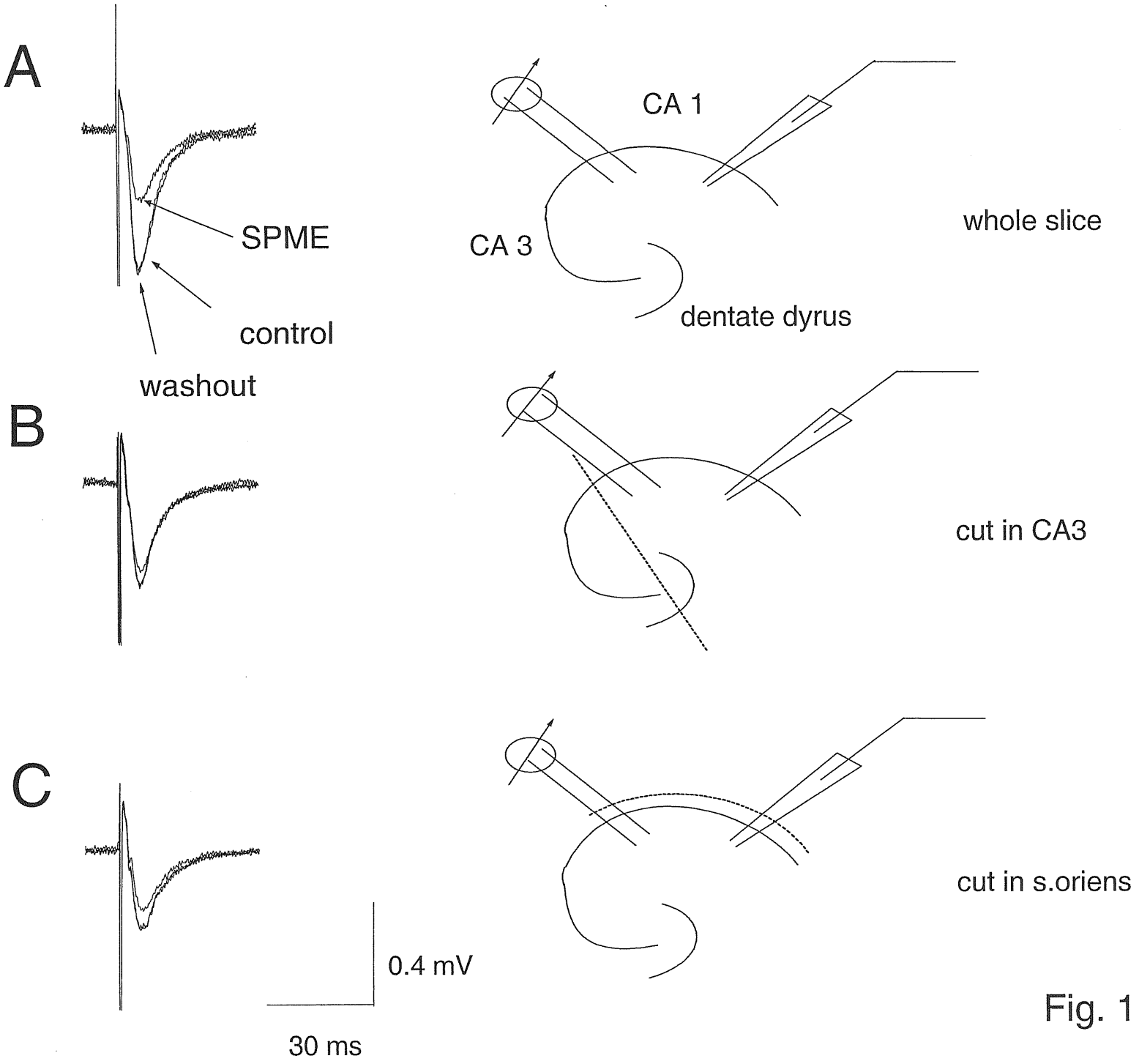

Fig. 11 page 54

Fig. 11 Depressant action of SPME in "mini-slices". A. In the whole slice preparation (schematically represented in the diagram on the right) SPME (0.1 μM) produced prominent depression of the field potential. B. Separation of the CA3 region with a sharp cut (dashed line) diminished the depressant effect of SPME. Note that the amplitude of the control field potential elicited by the stimulus intensity adjusted after the cut to produce the same ½ of the maximum amplitude of the response as before the cut in A, was diminished by 33%. C. After the additional cut in *SO* of the CA1 region the depressant action of SPME was not reduced anymore, whereas the amplitude was slightly decreased. Data from the same slice. Each trace is an average of 3.

against 45±9% for the field potential amplitude and 33±7% against 46±5% for the field potential slope). The remaining slice demonstrated a slight increase in the depressant response in SPME solution.

Attempts were also made to sever the connections between *SO* and CA1 pyramidal layer or between *SLM* and CA1 pyramidal layer. When either *SO* or *SLM* were ablated, the mini-slice became electrically silent. Thus, a section of *SO* was performed starting from the EC end towards the center of the CA1 (see example in Fig. 11C). The recording electrode was still kept in the dendritic field of the CA1 pyramidal layer in the area below the cut. A similar approach was used for severing the *SLM* from the pyramidal layer. Note that with either surgical manipulation there was a residual area of *SO* and *SLM* tissue, albeit distant from the recording electrode.

When cuts were made to eliminate the connections from *SO* or *SLM*, the amplitude of the control field potential was diminished by 41±7%, (n=9) and 24±8% (n=7), respectively, but the depressant effect of SPME on the residual field was not changed considerably. In fact, in slices in which *SO* was sectioned, SPME produced 30±9% depression in the field potential amplitude and 25±5% depression in the slope, whereas in the same prelesioned slices these values were 30±8% and 28±7%, respectively. When the horizontal cut was made along *SLM,* SPME elicited depression in the amplitude (46±10% against 42±9% in control) and in the slope (46±10% against 40±5% in control) of the field potential (n=7). In summary, after removal of CA3 or dentate gyrus areas the action of SPME was strongly decreased. Nevertheless, it was not feasible to assess the full contribution of *SO* and *SLM* areas because of the remaining components of those regions after surgical procedures.

It might be argued that that the smaller depressant action of SPME on synaptic transmission of "mini-slices" lacking CA3 or dentate gyrus region was the result of general tissue damage. Nevertheless, cutting the CA3 or dentate gyrus off did not apparently disturb the relatively remote CA1 area from which recordings were obtained. Any lesion of *SO* or *SLM*, which are very close to the site of microelectrode recording, should have been far more disruptive. In practice, control field amplitudes were diminished after surgical manipulation of *SO* or CA3 by 41 % or by 25 %, respectively. Despite the smaller field after severing the *SO* region the depressant effect of SPME was still present, whereas in slices with the ablated CA3 region (and better preserved field amplitude) the same peptide had a strongly reduced action.

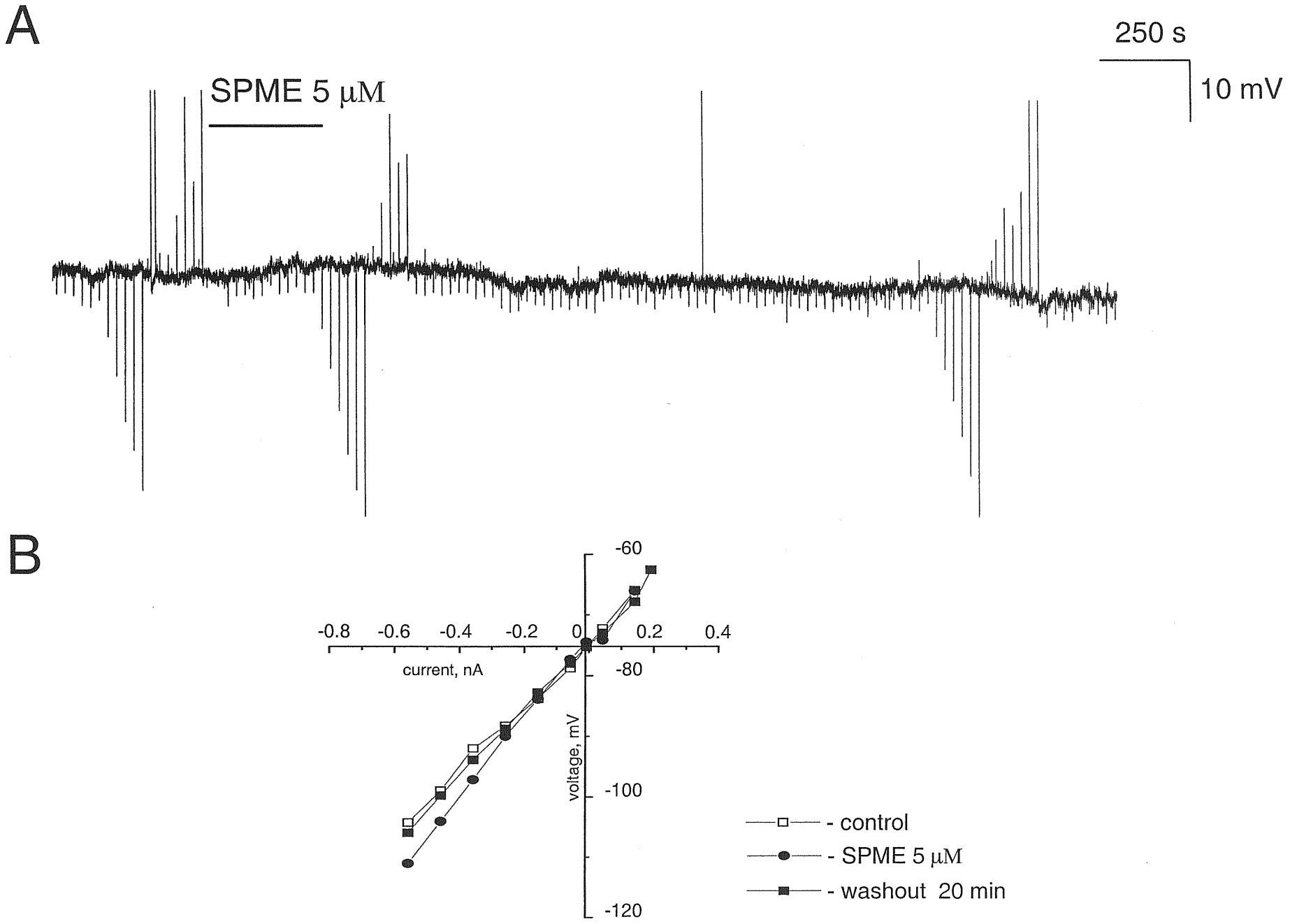

Fig. 12 page 57

Fig. 12. The effect of SPME on basic membrane properties of a pyramidal cell. A. Membrane potential trace indicating the response of the neurone to 5 μM SPME (horizontal bar). Current pulses were applied to generate I/V curves, like the one displayed in B. Note the slow and shallow (2mV) depolarization produced by SPME, reversible on washout. Recording done with K acetate electrode. RMP=-72 mV.

B. Current-voltage relations obtained before, during and after application of SPME. Note that in a region negative to resting membrane potential SPME reversibly increases the membrane input resistance, as shown by the steeper slope of the plot.

4.3. Effects of SPME on membrane properties of CA1 neurones:

In order to investigate the effect of SPME on basic membrane properties intracellular recordings with K-acetate or KCl filled electrodes were obtained from 59 neurons of the CA1 mouse hippocampal region. All cells had resting membrane potential (RMP) more negative than -55 mV and spike overshoot of 15-25 mV. The action potential duration at the base was 4.1±0.9 ms and 1.6±0.2 ms at 50% amplitude (n=17), that is larger than the values described by Lacaille (1991) for interneurones present in *St P* of the rat hippocampus (0.605±0.039 ms and 0.26±0.013 ms respectively). The afterhyperpolarization (AHP) evident after an individual action potential was smaller than the one typical for interneurones and on average was 4.2±0.6 mV (n=17; compare with -13.5±1 mV described by Lacaille, 1991). Pronounced spike-frequency adaptation was observed in response to intracellular injection of depolarizing current pulses (from 0.1 to 0.4 nA; 500 ms) in all neurones tested. These electrophysiological properties suggested that cells considered in this study were pyramidal neurones. Passive membrane properties in these cells (RMP=-70±2 mV; input resistance 36.4±6.6 MΩ; n=47) together with action potential parameters were comparable to those previously described for mouse neurones (Fournier and Crepel, 1984; Colling et al., 1996; Johnston et al., 1997).

When SPME (0.1-5 μM) was bath applied for 4 min, only slight changes in passive membrane properties of these neurones were evident. In 4/41 neurones (recorded with K acetate filled electrode) there was no change in RMP. In 14/41 cells a small depolarization of 1-3 mV took place (Fig. 12A) and in 21/41 cells a modest hyperpolarization (2-5 mV) was observed (Fig. 19A). Similar changes in membrane

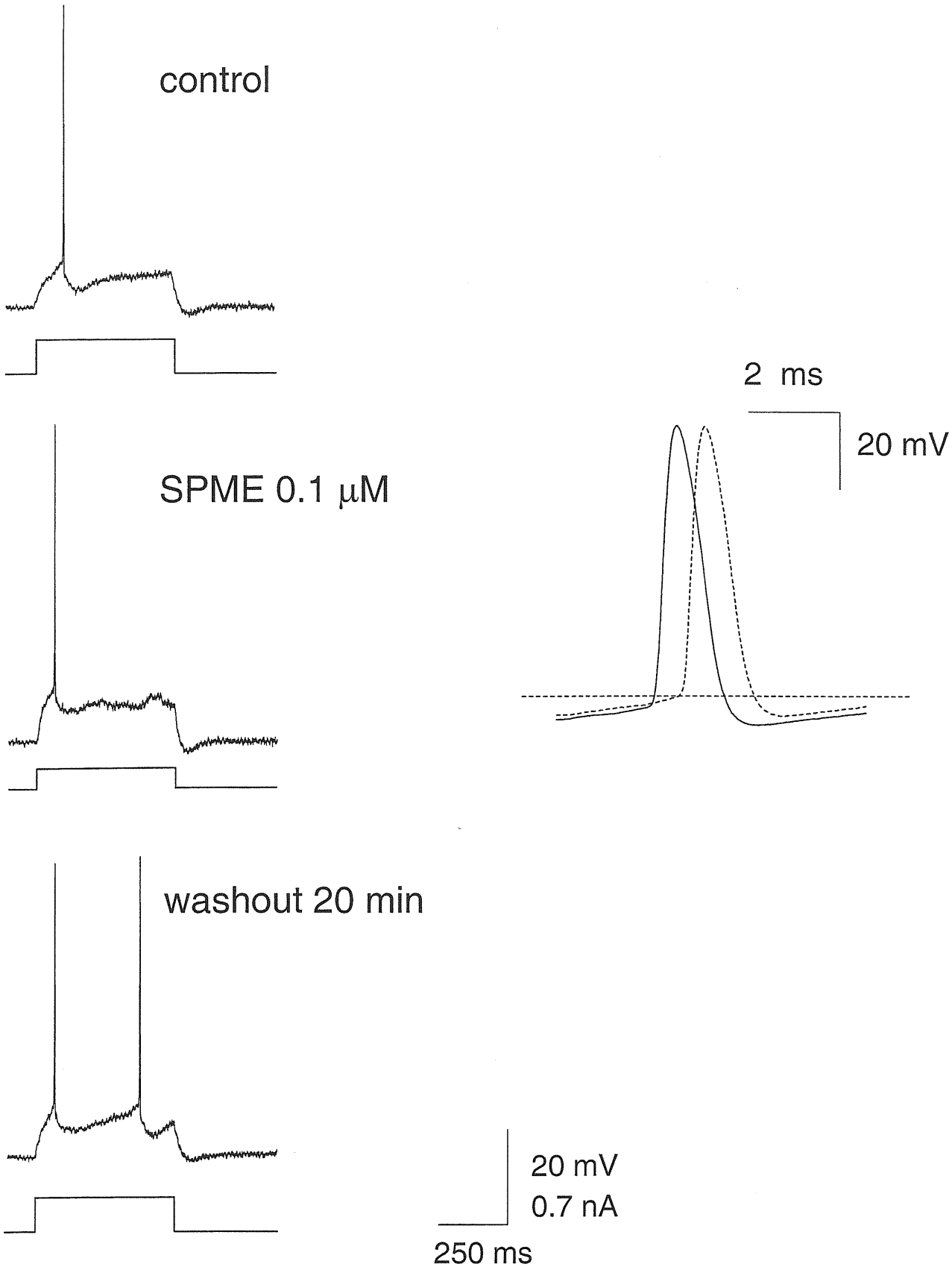

Fig. 13 page 59

Fig. 13. The effect of SPME on the excitability of a pyramidal cell. Intracellularly applied depolarizing pulses revealed a decrease in the spike current rheobase in SPME (0.1 μM) solution. Note that the latency of the first spike is also diminished. Insert on the right (trace from a different cell) shows change in spike latency in SPME (0.1 μM) solution (continuous line). Note also 3 mV decrease in spike threshold with respect to the dotted line in control (RMP=-67mV; n=5 for each trace;). Both recordings in A and B done with K acetate electrodes.

potential were observed in the presence of TTX (1 μM). Unlike the reproducible depressions of field potentials by SPME, several consecutive applications of this drug, even when separated with reasonably long periods of washout (30-40 min), could produced desensitization of the effect on membrane potential alteration (compare Fig. 19A with Fig. 19C). Nevertheless, application of different concentrations of SPME (0.1-0.5 μM; tested on different cells or on the same cell with long intervals for the washout) revealed a tendency for a dose dependent effect of the peptide on membrane potential changes. The lowest concentrations (0.1 μM) of SPME produced hyperpolarization in 80% of cases (28/35) cells, whereas the highest concentration (5 μM) induced hyperpolarization in 44% cases (4/9 cells) only. When two different concentrations were applied to the same cell (n=5) with 40 min washout in between, 0.1 μM SPME produced 3±1 mV hyperpolarization in all neurones, while the higher dose (1-5 μM) showed 3±0 mV depolarization in 3/5 and no change of membrane potential or 2 mV hyperpolarization in the remaining cells. Thus, the probability to observe membrane hypepolarization seems larger when the SPME was small.

Input resistance was not significantly (P>0.05) changed during SPME administration to the majority of neurons either in control solution (36.4±6.6 MΩ against 40.1±4.9 MΩ; 20/47 cells) or in 1μM TTX solution (38±7.8 MΩ against 41±7.8 MΩ; 3/6 cells). In the rest of cells recorded a reversible 29±4% decrease (19/47) or 25±6% increase (8/47) in cell input resistance was observed (see an example in Fig. 12B).

Intracellularly applied depolarising steps during SPME application to 75% of neurons revealed a decrease in spike current rheobase by 30±15 % (n=22). For the cell exemplified in Fig. 13 during SPME application the 0.2 nA step of current was sufficient

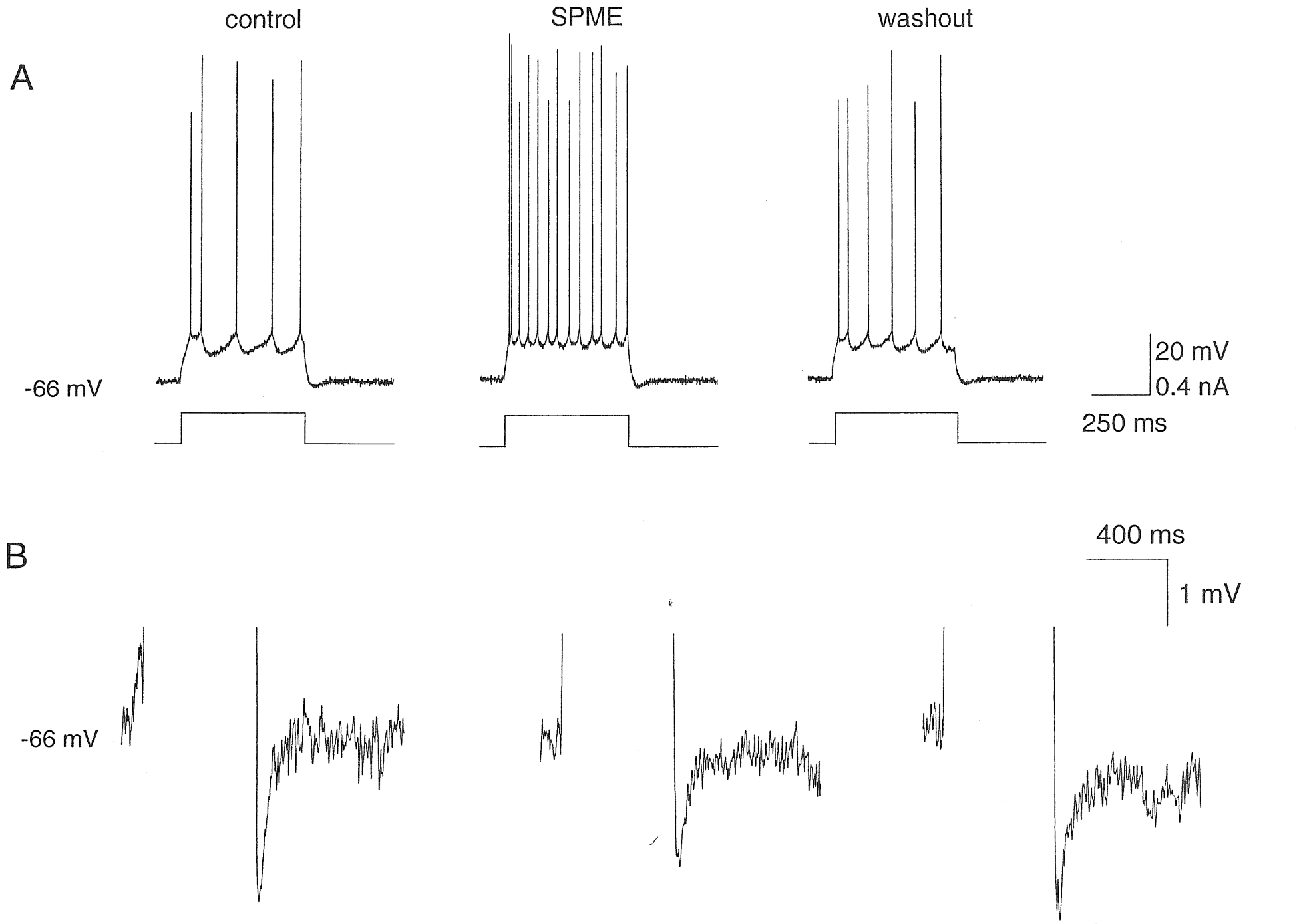

Fig. 14 page 61

Fig. 14. Changes in accommodation and AHP induced by SPME. A. Block of accommodation during application of 0.1 μM SPME. Note that, while the pulses of injected current are the same for all three panels (+0.2 nA, 500 ms, 0.04 Hz), in SPME solution the cell fires at higher frequency, twice than in control or during washout. B. Reduction in peak amplitude of the AHP after application of 0.1 μM SPME. Note that the step of current (0.2 nA in control and 0.3 nA in SPME; 500 ms, 0.04 Hz) produced the same number of spikes (truncated) in all three traces.

to evoke a single spike, whereas in control and washout 0.4 nA was required. In Fig 13 one can also note that the latency of the first spike was diminished during SPME application (111 ms versus 65 ms). This phenomenon was evident in 50% of cells. In 67% of cells tested during SPME administration the threshold of the spike was also decreased by 2±1 mV regardless changes in RMP produced by the peptide (see inset in Fig. 13, different cell).

Another phenomenon present in SPME was block of accommodation demonstrated in Fig. 14A. Note that in SPME solution (which in this particular cell did not produce any membrane potential change) the neurone fired more spikes in response to the same pulse of current (+0.2 nA). The accommodation index was evaluated by calculating the ratio between the first and the last interspike interval. For the cell in the Fig. 14A this index was greater in SPME and returned to the initial value upon washout. Although block of accommodation was evident in the majority of neurons (68%), enhancement of spike adaptation was seen in 21% of cells and in the remaining 11% of neurons no change in accommodation during SPME application was seen.

A reduction in peak amplitude of the AHP that followed a train of spikes elicited by a positive current injection was evident during application of SPME. An example is shown in Fig. 14B. Although the number of spikes (truncated) elicited by the current step is the same for all three panels, one can see that AHP was reversibly decreased by 31% in SPME solution (0.1μM; -2.9 mV versus -0.9 mV respectively). This depression of the AHP was seen in 60% of cells and on average of 35 neurones was equal to 2±1 mV. Although in Fig. 13 the AHP that follows the single spike was considerably reduced during application of SPME, the same did not seemingly apply to the AHP that appears at

the end of the current pulse. In a number of experiments when the effect of SPME was investigated on the AHP following various steps of current, it appeared that the AHP generated by intense stimuli (eliciting >5 spikes) was more sensitive than the AHP after small pulses (eliciting 1-4 spikes only). This differential sensitivity perhaps implies that certain distinct time- and voltage-dependent conductances were preferentially modulated by this neuropeptide. Although this aspect was not systematically addressed in the current study due to the limited number of cells (60%) displaying such an effect, whenever a consistent depression by SPME of the AHP was present, only strong intracellular stimuli (eliciting 5 or more spikes) were used to maximize the peptide effect.

Collectively, these results show that SPME had only slight and variable actions on resting potential and excitability of pyramidal neurons. The possible modulatory role of SPME on synaptic transmission was next investigated.

4.4. Both evoked EPSP and IPSP were decreased by SPME in pyramidal cells of hippocampus

The EPSP-IPSP response evoked by *SR* stimulation was recorded with K acetate filled microelectrodes in order to investigate the influence of SPME on the synchronous stimulus-induced release of glutamate and GABA. In Fig. 15A an example of these experiments is shown. The averaged EPSP-IPSP was observed in control solution for about 10 min. Afterwards 0.1 μM SPME was applied. At 3 min application a membrane potential hyperpolarization of about 2 mV was induced and was abolished by manual repolarization with +0.07 nA current injection to reveal that the EPSP and the IPSP were decreased (by 41% and 36%, respectively) regardless of the membrane potential change. After that the positive current was removed, the cell hyperpolarized by 3 mV until the end of SPME application, at that time the EPSP and IPSP were strongly reduced in amplitude (by 67% and 71%, respectively). With about 15 min washout full recovery of evoked PSPs from depression was obtained.

The amplitude of the control EPSP and IPSP was 5.1±0.4 and 1.1±0.1 mV, respectively (n=26, RMP=-69±1 mV). SPME (0.1-5 μM) reversibly lowered the amplitude of the EPSP by 39±6% in 69% cells kept at the same membrane potential as in control solution (while the EPSP was unaffected in 3% and increased in 28% cells). In 88% of neurones the IPSP amplitude was reversibly diminished by nearly the same degree (56±6%), whereas in 12% of cells IPSP increase was observed.

The amplitude of AMPA or NMDA induced depolarizations in TTX solution in the presence of SPME (0.1 μM) was tested in order to find out whether SPME changed the sensitivity of postsynaptic glutamate receptors. On the average of 3 cells (see Fig. 15B),

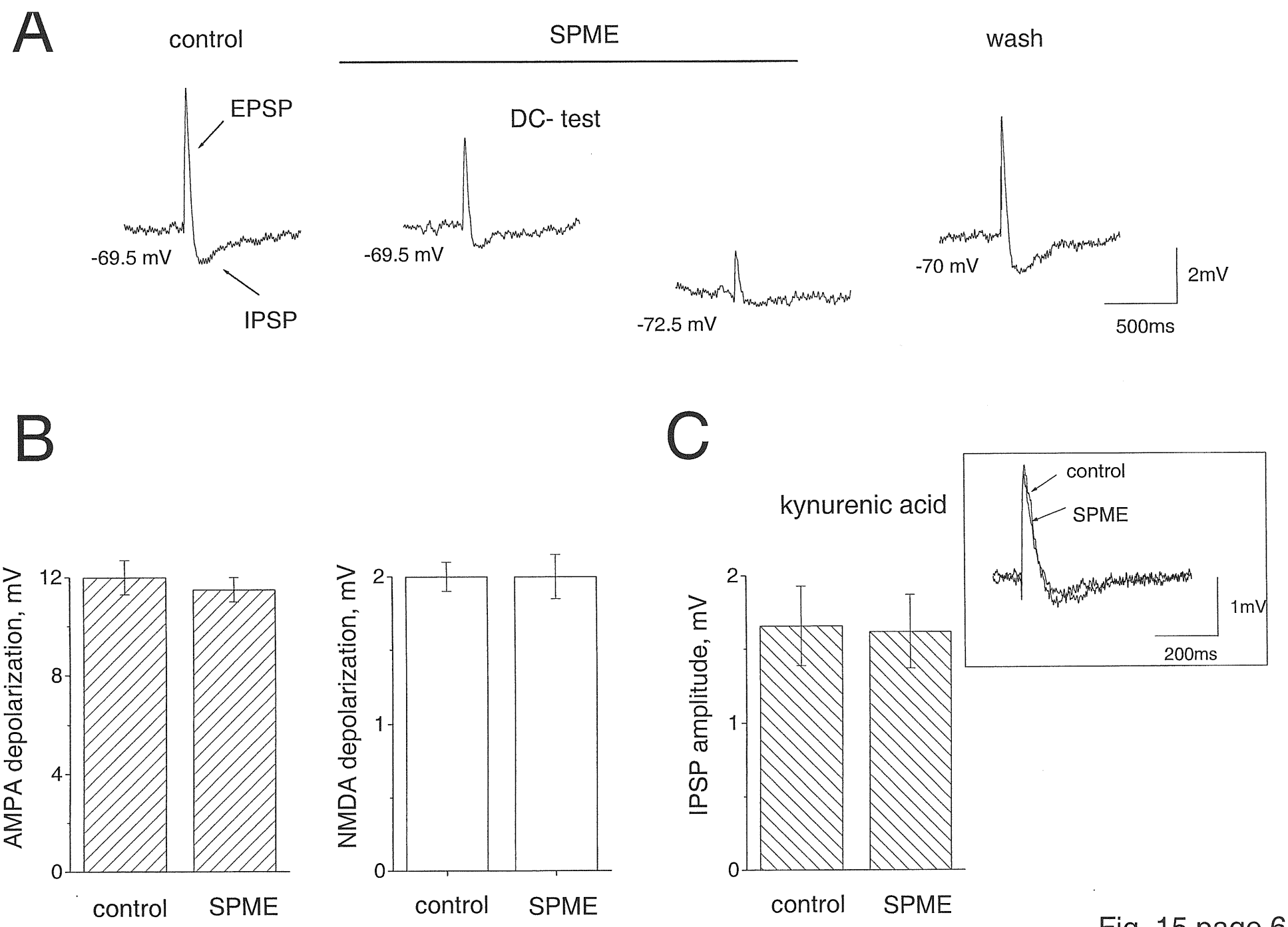

Fig. 15 page 65

Fig. 15. The depressant effect of SPME on evoked EPSP and IPSP is not exerted at postsynaptic level and requires a glutamatergic input to the IPSP. A: evoked EPSP-IPSP (arrows) complex in control, in the presence of 0.1 μM SPME (3 min application, DC-test, injected current=+0.07 nA), at the peak of action of the peptide (4 min application) or after 15 min washout. Note substantial reduction in amplitude of both potentials. The 3 mV hyperpolarization produced by SPME does not fully account for the IPSP decrease, as shown by preceding DC-test. Data are averages of ò3 responses. Intracellular recording with K-acetate electrode. B: histograms of depolarization produced by 4 μM AMPA (hatched bars) or by 10 μM NMDA (open bars) in TTX solution. No significant difference is observed in control *versus* 0.1 μM SPME containing solution. C: histograms of the amplitude of monosynaptic IPSP in control or in 0.1 μM SPME solution in the presence of 1 mM kynurenic acid (sample of original responses is shown in the inset; average of 10 traces). No change in the amplitude and shape of the events is observed. Data recorded with KCl electrodes from 5 cells.

virtually no change in the depolarizing action of AMPA or NMDA was observed when the neuropeptide was applied. Thus, these data made unlikely the fact that the EPSP was altered by a postsynaptic site of action of the peptide on glutamate receptors. The issue of whether SPME reduced the IPSP directly or indirectly remained unclear. Recording with K acetate electrodes, although providing the opportunity to monitor EPSP and IPSP simultaneously, did not allow detection of subtle changes in the amplitude of the IPSP due to its time course overlapping the one of the EPSP. Moreover, under these conditions the IPSP amplitude was inevitably small owing to the limited driving force for Cl^- which mediates this response (Sivilotti and Nistri, 1991). Thus, it seemed advantageous to investigate the IPSP with KCl

filled microelectrodes to increase its size by reversing the Cl^- gradient, after block of glutamate receptors with 1 mM kynurenate. The inset to Fig. 15C demonstrated this approach, which enabled to observe the monosynaptic IPSP as a large depolarizing response. Surprisingly, under these conditions SPME (0.1μM) failed to produce any detectable change in the amplitude of the IPSP (see also histograms in Fig. 15C). This finding indicates that SPME did not interact directly with the inhibitory GABAergic receptors localised postsynaptically on pyramidal neurones, but it modulated electrically induced IPSPs in a presynaptic manner and that this process required an intact glutamatergic drive.

4.5. SPME changed the frequency of spontaneous events on pyramidal neurones

In each neurone recorded intracellularly with a KCl-filled microelectrode spontaneous depolarizing postsynaptic potentials were observed. The frequency of such a spontaneous

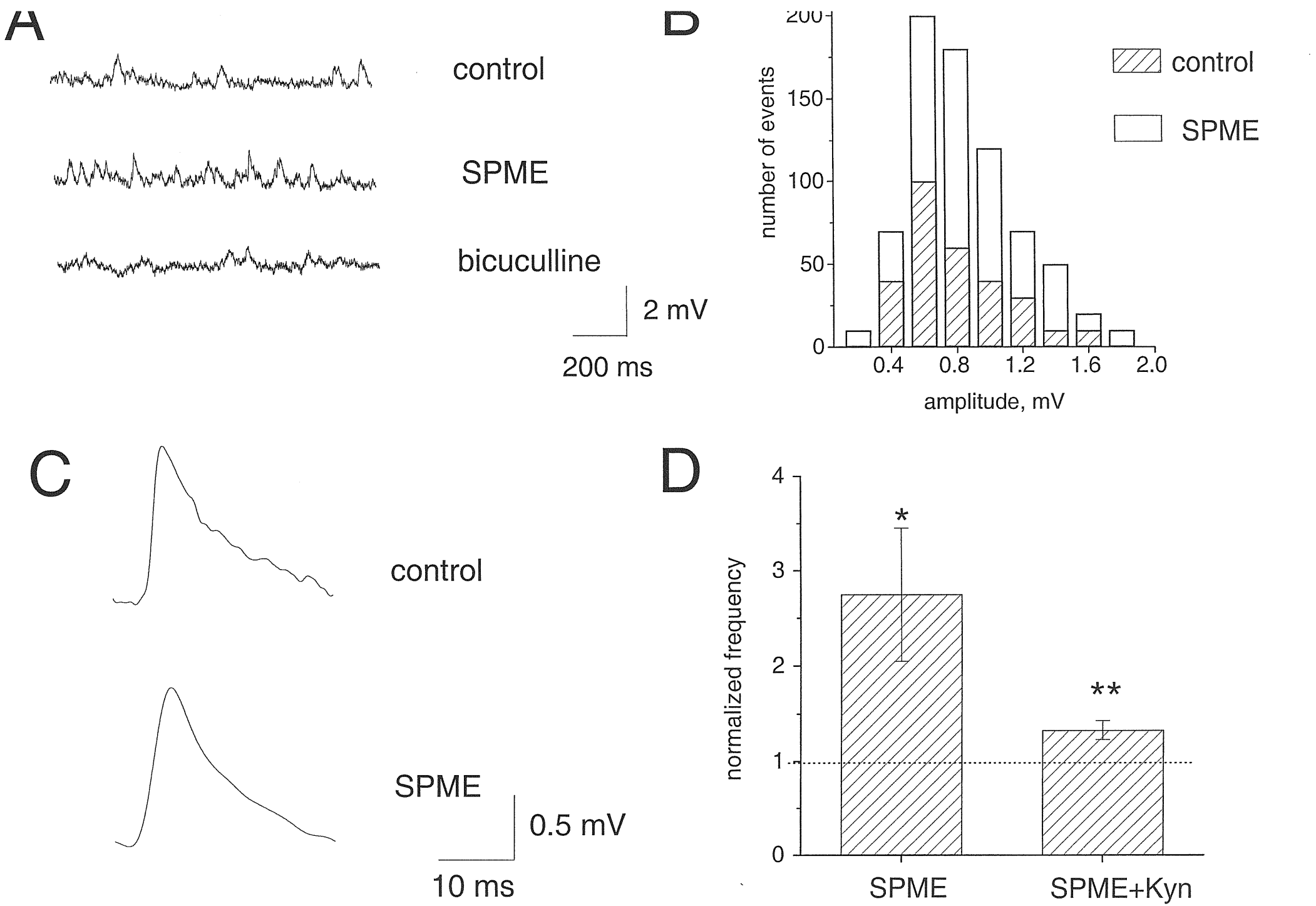

Fig. 16 page 67

Fig. 16. Upregulation of inhibitory spontaneous activity by SPME and its dependence on intact glutamatergic drive. All responses recorded with KCl electrodes. A: spontaneous activity in control solution, in 0.1 μM SPME solution or 15 min after superfusion with 10 μM bicuculline. In the presence of SPME note increases frequency of events which are mainly inhibitory since they are substantially reduced by bicuculline. B: amplitude histogram of spontaneous events in control (cross-hatched bars) or in 0.1 μM SPME solution (shaded bars). SPME largely enhances the frequency of spontaneous events of all sizes. The mean value is shifted to the left probably due to event overlapping. Same cell as shown in A. C: averaged spontaneous postsynaptic potential in control or in 0.1 μM SPME solution. Note similarity in amplitude and shape of the response (average of 100 events, same cell shown in A). D: histograms of spontaneous activity changes produced by 0.1 μM SPME in normal solution or after 1mM kynurenic acid application. The nearly three fold increase in frequency observed in normal solution is largely decreased in kynurenic acid solution. Dotted line represents control frequency value for standard and kynurenic solutions. Data (5 cells for each bar) are significantly different from their respective controls (*=$P<0.02$, **=$P<0.01$).

activity was increased by SPME (0.1 μM) in 12/16 cells from 3±0.7 to 9.9±4.3 s^{-1} (see example in Fig. 16A, RMP=-66 mV). The time course of these changes was analogous to the one of the depression of electrically evoked synaptic transmission. Pretreatment with the NK_1 receptor antagonist SR 140333 (4 μM) for at least 20 min prevented this change (2.0±0.4 *versus* 2.2±0.3 s^{-1}, n=5). These spontaneous events recorded in control solution were presumed to be mainly mediated by $GABA_A$ receptors as bicuculline (10 μM) greatly reduced this activity (Fig. 16A). Thus, SPME appeared to increase the frequency of spontaneous IPSPs. The neuropeptide largely enhanced the frequency of spontaneous events of all sizes, as it is evident from the amplitude histogram plotted in Fig. 16B. While the mean value of the histogram in Fig. 16B was shifted to the right, the average amplitude of the events did not change as shown by the similar size and shape of spontaneous responses recorded before and after application of SPME (Fig. 16C). The histogram shift might have been due to the increased number of the overlapping events that took place in SPME solution.

The spontaneous event frequency tested in slices superfused with 1 mM kynurenic acid throughout the experiment (1.8±0.2 s^{-1}; n=5) was not significantly different from the one observed in control solution ($P>0.05$). However, the frequency increase produced by SPME was much smaller than the one found in control solution, albeit still statistically significant (Fig. 16D).

In experiments conducted with K-acetate microelectrodes spontaneous EPSPs and IPSPs had opposite polarity and thus allowed simultaneous monitoring of their sensitivity to SPME at resting membrane potential without any pharmacological separation. However,

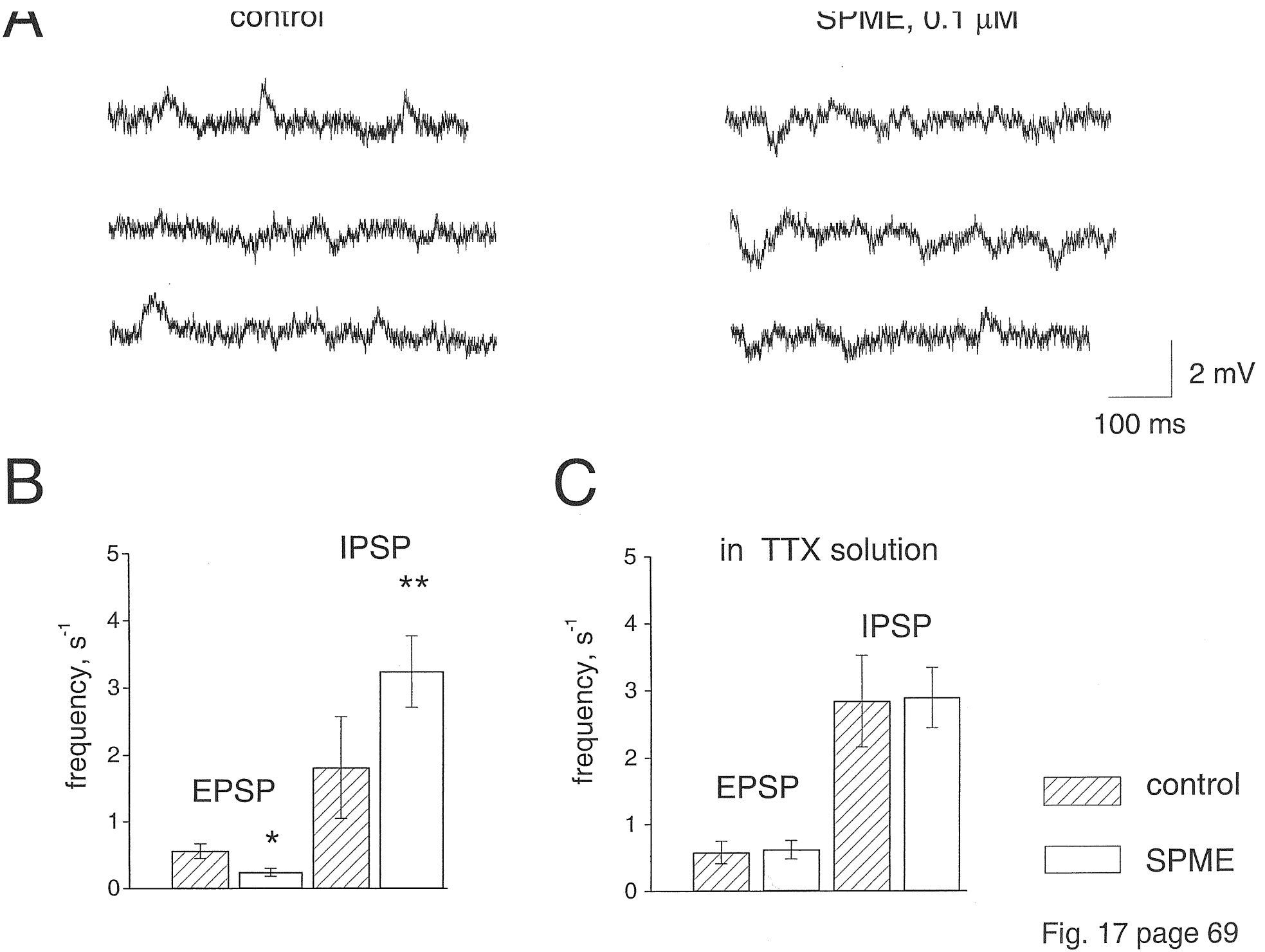

Fig. 17 page 69

Fig. 17. Changes in excitatory and inhibitory spontaneous activity produced by SPME in normal and TTX containing solutions. All results are with K-acetate electrodes. Hatched bars represent data in control solution while open bars indicate those in SPME A: EPSP and IPSP frequency in control or in 0.1 μM SPME solution. Whereas IPSPs are increased in frequency, EPSPs frequency is decreased. *=$P<0.02$, **=$P<0.03$; n=5 cells. B: frequency of EPSPs and IPSPs in control solution or following application of 0.1 μM SPME. TTX (1μM) is present throughout. Since no significant difference in frequency of events is observed in TTX solution, the target for the peptide reduction in synaptic transmission is presumably the action potential dependent release (cf. also Miles et al., 1996). Data from 4 cells.

these experiments were limited in their resolution because small amplitude events were probably included in the background noise as it is evident in the cell exemplified in Fig. 17A. In 5 cells (Fig. 17B; RMP=-68±1 mV) SPME (0.1 μM) increased IPSP frequency from 1.8±0.7 s^{-1} to 3.2±0.5 s^{-1} (P<0.02), but it reduced EPSP frequency from 0.56±0.11 s^{-1} to 0.24± 0.06 s^{-1} (P<0.03). Conversely, there was no significant alteration in the amplitude of EPSPs (1.4±0.1 mV *versus* 1.3±0.1 mV in control) or of IPSPs (0.6±0.1 mV *versus* 0.6±0.1 mV in control). In TTX containing solution no change in the frequency of spontaneous depolarizing or hyperpolarizing activity was, however, elicited by SPME (Fig. 17C; n=4; RMP=-69±1 mV). On the same cells in TTX solution, the amplitude of excitatory events was unaltered in control or SPME solution (1.1±0.1 mV in either case) and no amplitude change was detected for inhibitory events before or after SPME application (0.5±0.1 mV in either case).

In addition to its ability to modulate glutamatergic and GABAergic transmission, SPME was tested also on cholinergic responses either induced by an exogenously applied agonist or by electrical stimulation of ACh-releasing nerve fibres.

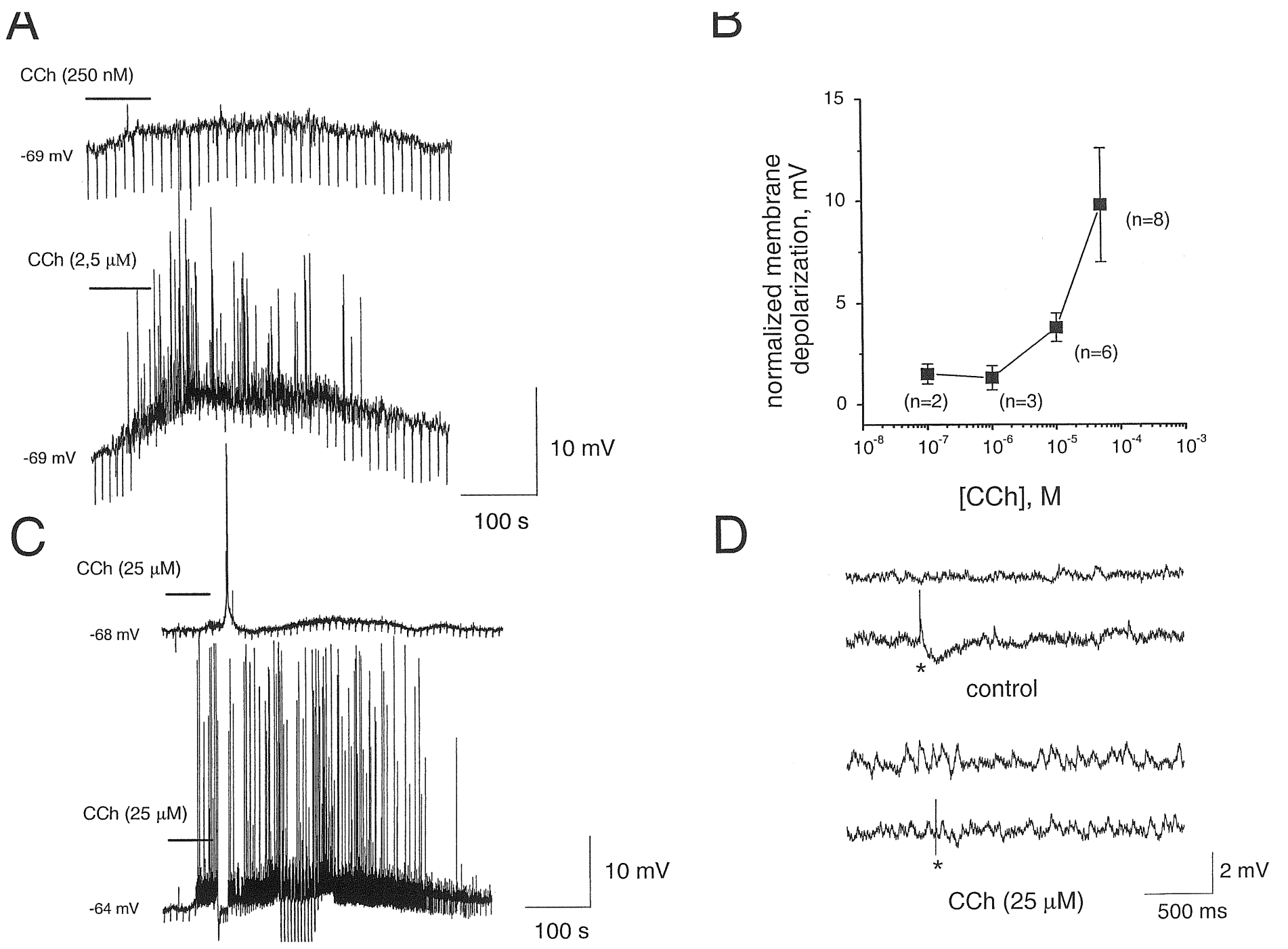

Fig. 18 page 71

Fig. 18. Characteristics of the action of CCh on hippocampal neurones. A. Membrane potential traces demonstrating main effects produced by brief applications of CCh (horizontal bar) and their concentration dependence. Both recordings obtained from the same cell with K acetate electrode. Downward deflections represent electrotonic potentials elicited for the monitoring cell input resistance changes.

B. log concentration-response curve for the depolarizing action of CCh. The value of the maximum response was not obtained as only small responses were used for the present study.

C. Membrane potential traces showing voltage dependence of the responses to CCh. Both recordings with K acetate electrodes from the same cell. Downward deflections are generated in response to current pulses of various amplitudes applied to construct I/V curves and to monitor changes in resting input resistance of the cell.

D. oscillatory activity induced by CCh (bottom traces). Note that evoked EPSP-IPSP complex, evident in control (top traces), is blocked in CCh solution (marked with asterisks). Recordings at a greater scale from C (top trace) prior to and 2 min after CCh application.

4.6. Effects of carbachol application

Bath application of the cholinergic agonist carbamylcholine chloride (carbachol, CCh) for 1-1.5 min (in the range of concentrations between 0.1 μM to 100 μM) produced large excitation of CA1 pyramidal cells. Fig. 18A shows an example of the most common actions elicited by CCh such as membrane potential depolarisation, increase in spontaneous synaptic activity (evident as an increased background noise) and induction of repetitive firing of the cell. This response to CCh was concentration-dependent (see Fig. 18A). Fig. 18 B shows that on a sample of hippocampal neurones the amplitude of membrane depolarization by CCh was dose-dependent for equivalent values of resting membrane potential. The occurrence and magnitude of depolarizing responses to CCh were also membrane potential dependent. In fact, in Fig. 18C the same concentration of CCh (10 μM) produced a slight depolarization (3 mV) when the cell was kept at -68 mV resting potential. Note however that the response was increased threefold when CCh was applied at -64 mV, and was accompanied by robust spontaneous activity and spike firing. Since for the present experimental protocols we needed moderate responses to CCh, cells were maintained at negative resting membrane potential (RMP -70±2 mV) by injecting hyperpolarizing current whenever necessary. In general, CCh (0.1 μM-100 μM) application elicited cell membrane depolarisation in 87% of cells (34/39) which was on average 6±2 mV. In a minority of cells (5/39, RMP=-71±3 mV) CCh evoked prominent membrane hyperpolarisation (7±2 mV) which in 4 cells was followed by sustained 1 or 2mV depolarisation. Changes in membrane potential appeared at the end of CCh application and were variable in their duration but usually reached their maximum two-five min later and did not last more than ten minutes. Several repeated applications of

CCh interrupted by 20 min washout period did not reveal any desensitisation of the response. In the present experiments carried out in ACSF CCh depolarisation was not associated with any significant change in the cell input resistance at rest level, measured during the DC-test applied at the second min of washout (47±12 MΩ versus 46±12 MΩ). This fact, together with very modest changes in membrane potential, probably was due to more negative RMP of the neurons in our study with respect to those in previous reports, which found a resistance increase (-70-71mV against -60-65 mV; Cole and Nicoll, 1984; Pitler and Alger, 1992). Since a decrease in several voltage dependent K^+ conductances has been implied to mediate the principal muscarinic effects (Krnjevic et *al.*, 1971; Benardo and Prince, 1982; Cole and Nicole, 1984), the fact that the cells of the present study had more negative resting potential (and thus probably a lesser degree of K^+ conductance activation) may account for the relative lack of changes in resting input resistance during application of CCh.

A prominent feature of the response induced by CCh was the change in on-going baseline activity. In the example of Fig 18 A (bottom trace) when the cell was bathed in 2.5 μM CCh solution (note that 250 nM CCh was near threshold for responsiveness; top record of same Fig 18 A) there was intense discharge of depolarizing events which often reached threshold for firing action potentials. On a pool of 7 cells the average frequency of depolarizing events went from 0.7±0.2 Hz in control to 2.7±0.7 Hz in 25 μM CCh solution. During a large response to CCh the complex nature of these events (which were often overlapping in time) precluded their reliable analysis in terms of frequency and amplitude. When the action of CCh in terms of membrane depolarization was smaller (see example in Fig. 18 C, top trace at -68 mV resting potential, different cell from Fig.

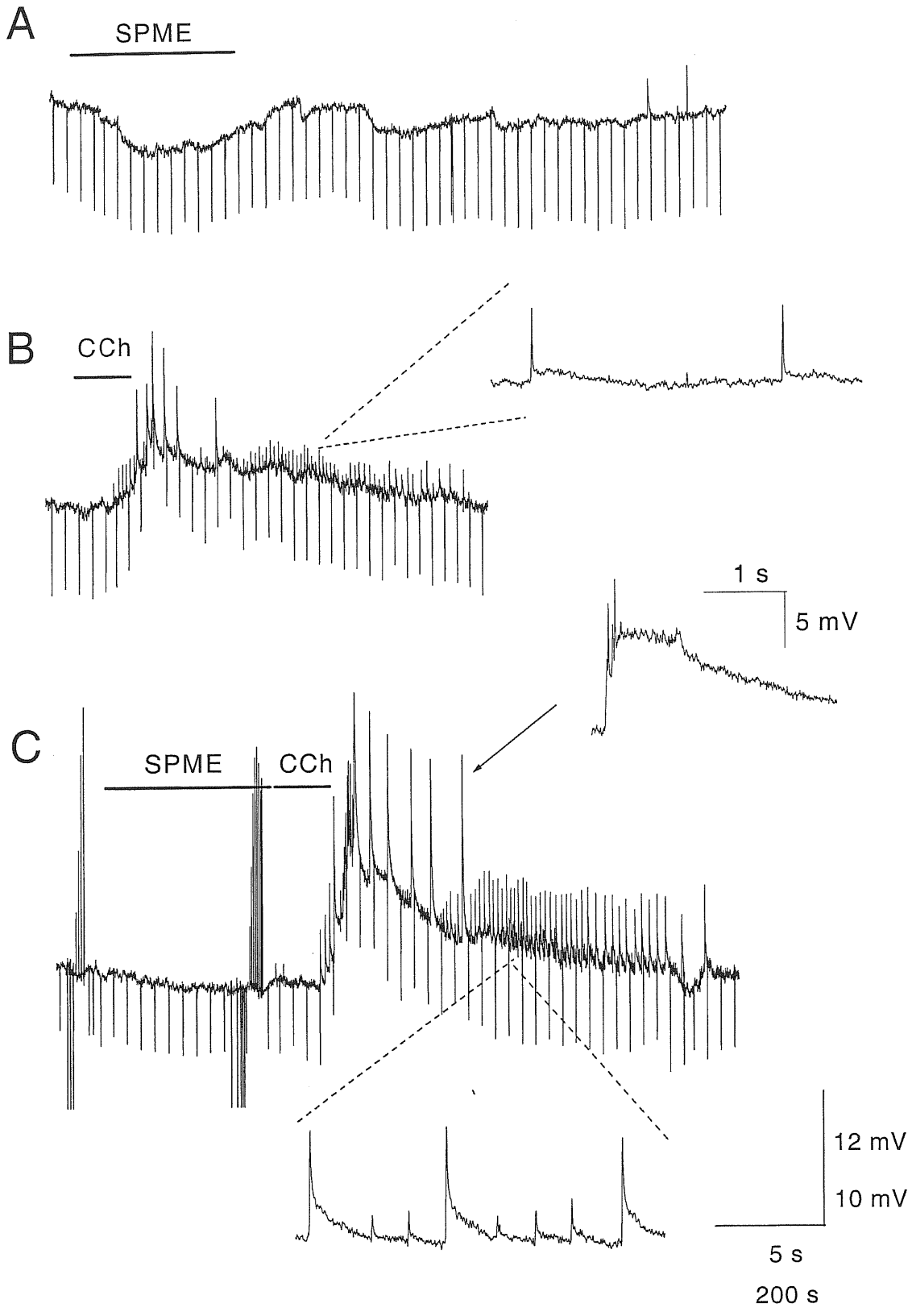

Fig. 19 page 74

Fig. 19. Pre-treatment with SPMP potentiated the excitatory effect of CCh.

A. Membrane potential trace demonstrating the hyperpolarizing (5 mV) effect of a single application of 0.1 μM SPME (horizontal bar) with recovery upon washout. Note that membrane potential return to baseline started when the compound was still present in the bath, thus indicating response tachyphylaxis.

B. Membrane potential trace illustrating the effect of single application of 25 μM CCh. Note sustained depolarisation of 5 mV with bursting activity at its peak and rapid discharges during repolarization.

C. Membrane potential trace demonstrating potentiation of the response to CCh by preceding application of 0.1 μM SPME. Note the 12 mV depolarization and threefold increase in spontaneous bursting activity and rapid discharges (see expanded record in inset). Note that the initial hyperpolarizing response to the neuropeptide (5 mV) was greatly desensitised during this second application (1.5 mV) separated by 40 min washout (compare with A). All traces are from the same cell (RMP=- 72 mV). Downward deflections and upward deflections during SPME application in C represent responses to current pulse injections applied to monitor changes in input resistance or to generate I/V curves.

18 A), -68 mV resting potential, different cell from Fig. 18 A), a single, large discharge (with superimposed spikes) emerged and was followed by a period of intense, low amplitude on-going activity which is shown on a much faster time base in Fig. 18 D. In this case low amplitude depolarizing events presented an oscillatory nature in analogy with the phenomena amply described by previous investigators (Pitier and Alger, 1992; Behrends and Bruggeencate, 1993). These oscillations require intact synaptic inhibition as they are blocked by bicuculline or picrotoxin (Pitier and Alger, 1992; Behrends and Bruggencate, 1993) as confirmed in the present experiment (data not shown). Note also that a single electrical stimulus, which in control solution evoked a typical EPSP-IPSP sequence, was unable to induce a response in the presence of CCh (compare traces with asterisks in Fig. 18 D). On average the reduction of the EPSP was 76±11 % while the reduction of the IPSP was 81±10 % (n=15). On the same cell shown in Fig. 18C a small depolarization of the resting potential (4 mV) largely potentiated the excitatory action of CCh which was then characterised by intense discharge activity.

Fig. 19 shows, in addition to SPME (Fig. 19 A) and its interaction with CCh (Fig. 19 C), further examples of the membrane response characteristics to CCh. In particular, when CCh (25 μM) was applied (Fig. 19 B) there was a membrane depolarization with large spontaneous depolarizing events at its peak. During the gradual return of the membrane potential to control level a periodic type of oscillatory activity emerged. Examples of these responses are shown at higher gain/speed in the inset alongside Fig. 19 B. These events, which persisted for several seconds at a frequency of 0.03 Hz and slowly disappeared when the cell regained its resting potential, have been observed before in the rat hippocampus (MacVicar and Tse, 1989; Williams and Kauer, 1997) These responses

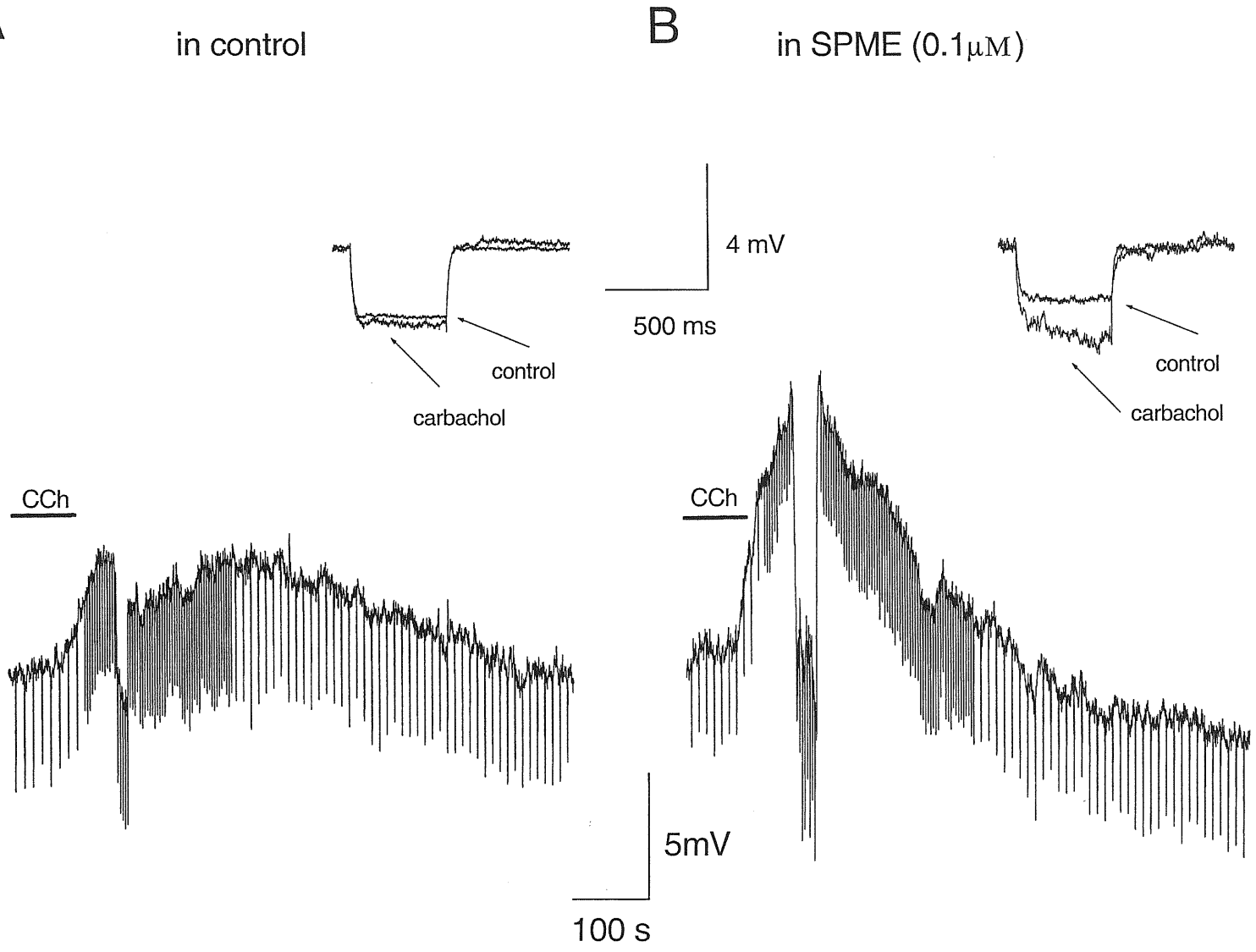

Fig. 20 page 76

Fig. 20. Potentiation by SPME of the response to CCh in TTX solution. A. Depolarization of 6 mV produced by 25 μM CCh application (horizontal bar) in the presence of 1 μM TTX. Note an increase in cell input resistance evident from augmented electrotonic potentials obtained during DC-test applied at the peak of the response (see inset, n=5) B. Response to CCh is greatly enhanced by SPME: 11 mV depolarization (183% increase versus control) and 92% increase in input resistance in DC-test at the depolarization peak (see inset, compare with A). In this cell SPME produced 1 mV depolarization compensated for by injection of positive current (0.04nA). Note also a 20% decrease in input resistance produced by SPME (compare with initial downward deflections and electrotonic potentials in insets in A). A, B recorded with K acetate from the same cell, RMP=-66 mV.

are considered to be due to network synchronization evoked by CCh at CA3 level. This synchronous discharge is thought to be then projected to the CA1 region where it can be detected by intracellular recordings from CA1 cells like in the present experiments. In the present series of experiments these persistent oscillations appeared in 11/36 cells exposed to CCh and pharmacological tests indicated that no rapid discharges were present when kynurenic acid (1 mM, n=2), CNQX (10 μM, n=3), or TTX (1 μM) was applied to the bathing solution.

Blocking oscillatory events with TTX allowed an analysis of the direct responsiveness of CA1 neurones to CCh. Among 16 cells tested in TTX (1μM) 81% showed a depolarising response to CCh (4±1 mV, RMP=-70±1 mV, see example in Fig. 20A). The remaining responses were hyperpolarising (6±2 mV), one of which was preceded by a 2 mV depolarisation. During application of CCh in TTX solution an increase in cell input resistance (15±3%) was revealed in 50% of neurones (8/16; see inset to Fig. 20A). No increase in background spontaneous activity nor appearance of bursts was evident.

The effects produced by CCh were blocked by pirenzepine (0.5 μM; n=2) or atropine (10 μM, n=3) in a reversible or irreversible manner, respectively (see Fig 21A,B). These data were in agreement with previous studies on the excitation produced by ACh and CCh on the rat hippocampal neurones (Benardo and Prince, 1982; Cole and Nicoll, 1984; Pitler and Alger, 1992; Bouron and Reuter, 1997; Psarropoulou and Dallaire, 1998).

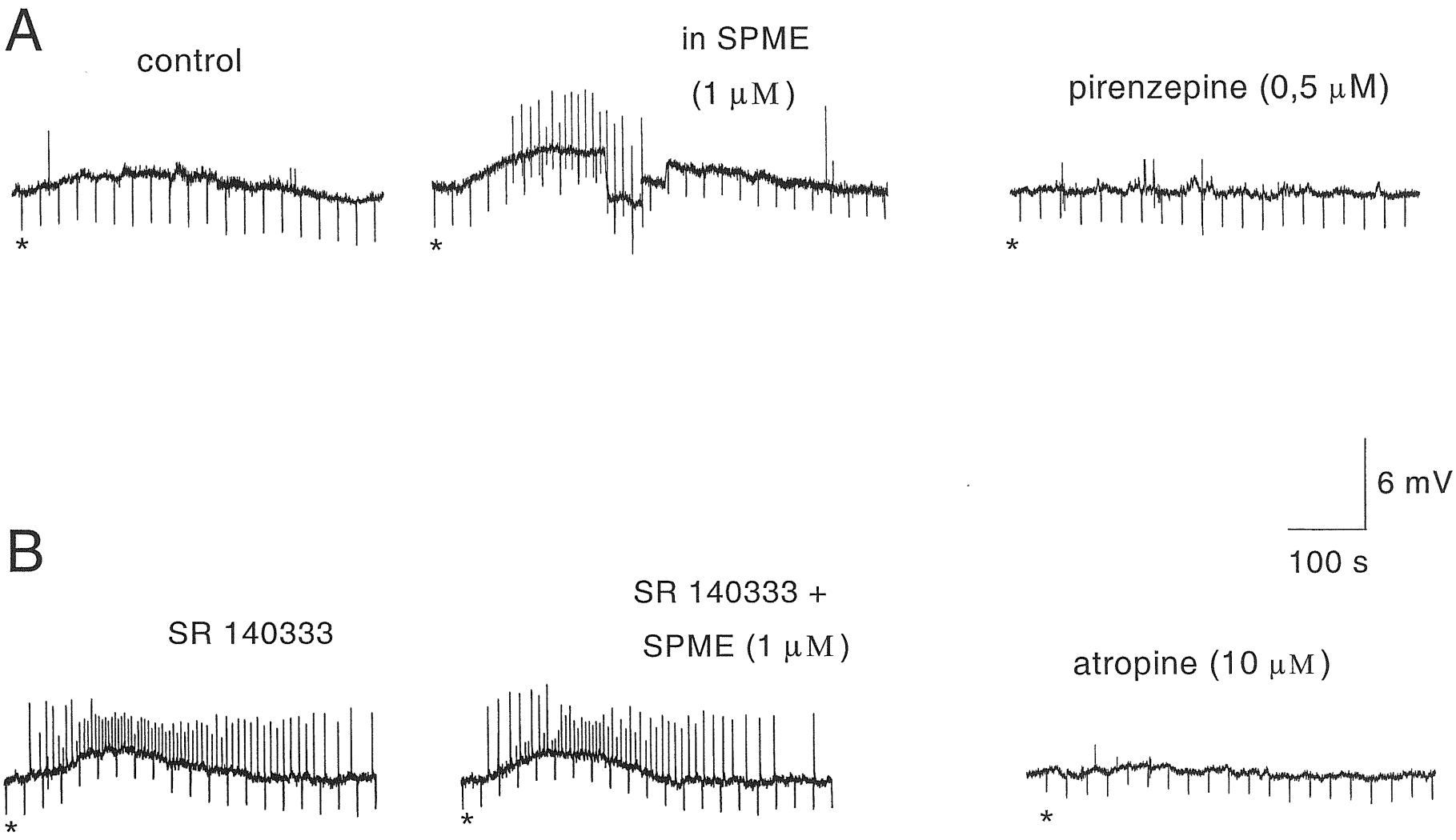

Fig. 21 page 78

Fig. 21. Effects of SPME, pirenzepine, atropine and an NK_1 receptor antagonist on the response evoked by CCh. A. A single application of 25 μM CCh for 100 s (asterisk) produced a shallow 1.5 mV depolarization with a small increase in spontaneous activity evident as background noise. Application of 1 μM SMPE elicited a 3 mV hyperpolarization, that waned at the 4 min of application (desensitization, this part of the trace is not shown). The subsequent administration of 25 μM CCh (asterisk) revealed an enhanced depolarization (3.5 mV), increased background noise and brought about synchronous rapid discharges at about 0.12 Hz rate, present during manual hyperpolarization of the cell. Pre-treatment with pirezepine (0.5 μM, 6 min) abolished both effects of CCh on membrane potential and spontaneous activity. B. Application of 25 μM CCh (asterisk) in the presence of 4 μM SR 140333 (30 min) produced a 2 mV depolarization and synchronous rapid discharges at the rate of 0.08-0.2 Hz. Pre-incubation with SPME did not alter these effects of CCh anymore (see middle trace, 2 mV depolarization, bursting rate 0.08-0.2, compare with middle trace in A). CCh applied in 10 μM atropine solution (7 min) produces no evident effect. A and B are from the same cell recorded with K acetate electrode.

4.7. Modulation by SPME of the response to carbachol

In this set of experiments the effect of CCh was tested in the presence of SPME. In accordance with our recent experiments, application of 0.1 μM SPME to a sample of 30 cells produced only moderate changes in basic membrane properties. 75% of neurones were slightly hyperpolarised (by 2-5 mV, see Fig. 19A), while in 25% of cells a small depolarisation (2-3 mV) took place. In the majority of experiments membrane input resistance remained unchanged during the neurokinin application. Only in a small percentage of neurones slight input resistance alterations were noted (decrease by 15±3% in 7/30 cells and increase by 11±1% in 3/30 cells), regardless of membrane potential variations.

Notwithstanding such small action of SPME per se, the excitatory response to CCh became dramatically enhanced after neurokinin application. As shown in Fig. 19C the amplitude of cell depolarisation evoked by CCh (25 μM) was seven-times larger and the spontaneous synaptic activity was increased threefold with respect to the control CCh test (compare with record in Fig. 19B). Although the total duration of the response to CCh was not particularly changed, the area calculated as an integral of membrane potential deviation from the baseline was 184% of the previous response. Out of 21 cells tested in ACSF with such a protocol, 65% neurones demonstrated a similar potentiation of the initial response to CCh. Cell membrane depolarisation produced by CCh (0.1 μM-100 μM) was increased by 129±56% ($P<0.001$) and the area of the response was augmented by 98±45% ($P<0.02$). During the DC-test performed at the peak of membrane potential depolarisation (usually 2-3 min after CCh application), 30% of neurones demonstrated a

Carbachol induced bursting activity

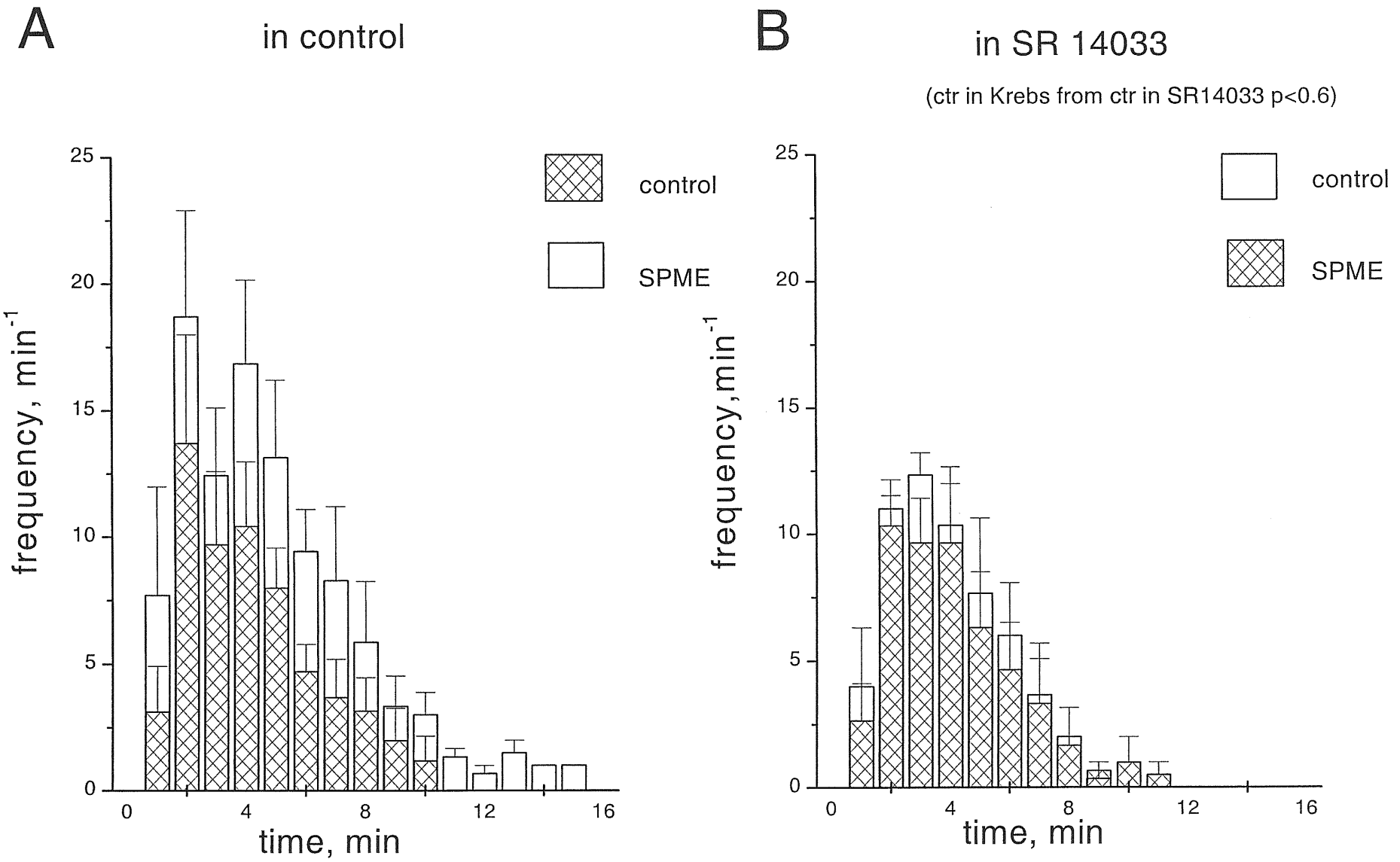

Fig. 22 page 80

Fig. 22. CCh- induced bursting activity. A. Frequency histogram of spontaneous discharge activity evoked by CCh in control solution before (hatched bars) and after (open bars) application in SPME. In these measurements both large bursts and rapid discharges were included. Note an increase in frequency and duration of all these events in SPME. B. Frequency histogram of discharges in the presence of the NK_1 antagonist SR140333 (4 μM) during single application of CCh (open bars) and after SPME pre-treatment. There is no significant difference in the two conditions. Zero time is the moment of application; pooled data from 11 cells before and after adding SR 140333.

rise in input resistance by 26±5%, whereas in the remaining cells there was no change. In those cells on which CCh evoked a hyperpolarizing response, SPME decreased the initial hyperpolatization by 30±15% (n=3), it increased it by 25% in one case and left it unchanged in another cell.

The potentiating action by SPME was not limited to membrane potential or input resistance changes. In fact, there was also strong facilitation of the number of large bursting events appearing at the peak of the response (one of them is shown at higher gain, see arrow in Fig. 19C). Additionally, during the slow repolarization of membrane potential after CCh the cell entered into a repetitive oscillatory activity (0.1 Hz; compare it with trace in Fig. 19B). The variable nature of these membrane oscillations is depicted at higher gain in the bottom record of Fig. 19C. In the presence of CCh the frequency of all bursting events (near the depolarization peak as well as during the repolarization phase) was calculated taking as zero time the CCh application start and compared with the responses evoked by CCh after pretreatment with SPME. These data are summarized in Fig. 22A (n=11 cells) in which it is clear that oscillations of various frequency were uniformly accelerated by SPME which also led to a more persistent phase of slow oscillatory activity lasting more than ten min after CCh washout. In 4 cells SPME pre-application actually brought about the appearance of this type of spontaneous events. The inhibitory effect of CCh on evoked postsynaptic potentials was also present in SPME and, in terms of amplitude, was not notably affected (80±12% versus 74±9% in control for EPSP and 81±9% versus 78±6% in control for IPSP; n=11).

The potentiating effect of SPME pre-application on CCh responses was present in 50% of cells tested in TTX solution (see an example of such recording in Fig.20A,B). Membrane

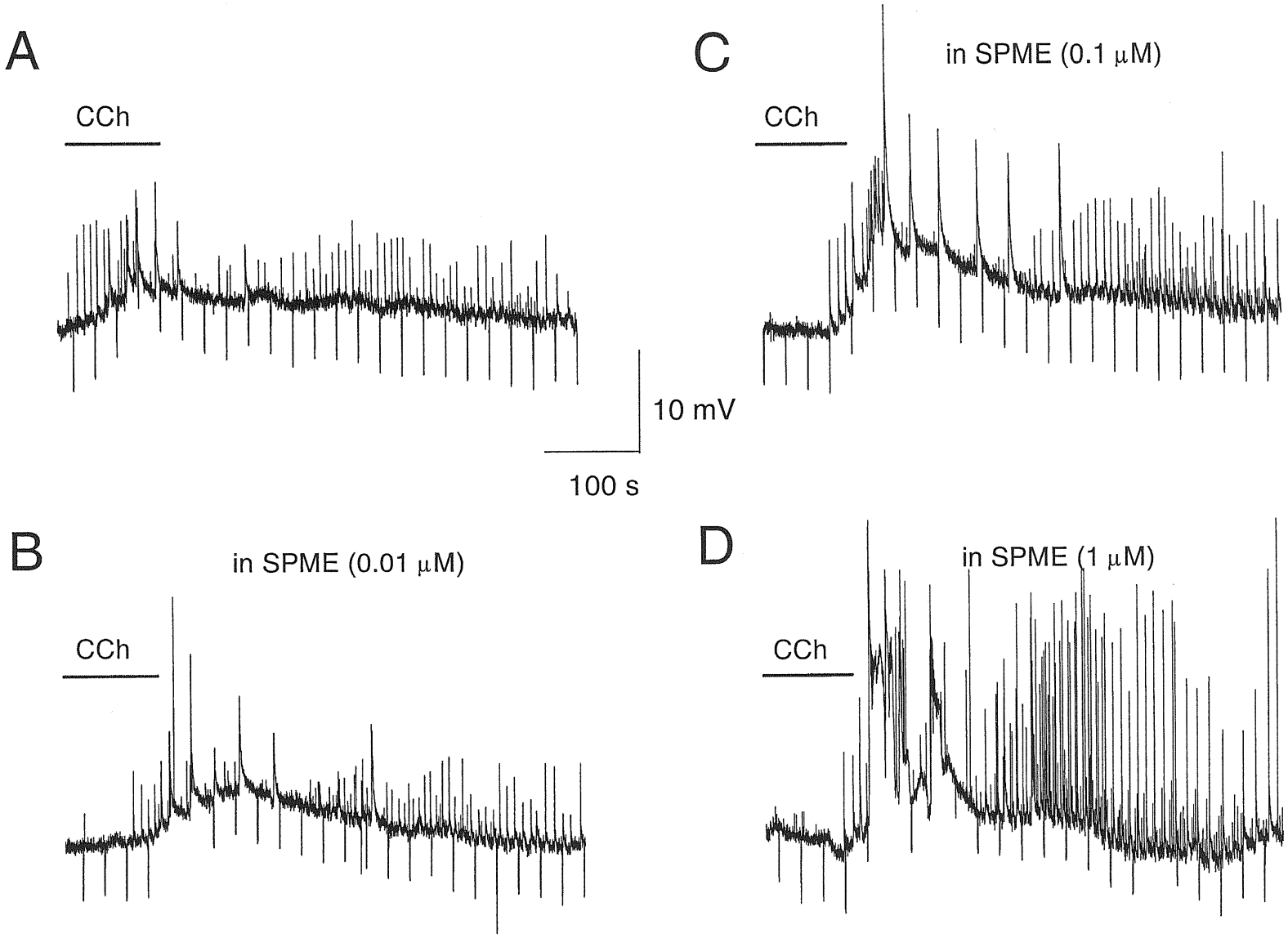

Fig. 23 page 82

Fig. 23. The SPME potentiation of CCh response was concentration dependent. A. Membrane potential trace of a single application of 25 μM CCh (horizontal bar). Note CCh evoked a moderate depolarization (5 mV) and an increase in synchronous bursting activity. B. 0.01 μM SPME slightly enhanced CCh induced depolarization (7 mV) without prominent changes in spontaneous activity. C. 0.1 μM SPME pre-treatment significantly increased effect of CCh both on depolarization and frequency of spontaneous activity; D. Very strong potentiation of excitatory response to CCh produced after application of 1 μM SPME. All traces are from the same cell. Applications of CCh are separated with at least 20 min washout period. Recordings done with K acetate electrode. RMP=-66 mV.

potential depolarisation and the area of the response were increased on average by 83±14% (P<0.01) and 60±11% (P<0.02), respectively (n=15, RMP=-70±1mV). Input resistance in 50% of these cells was increased by 46±25% (see Fig. 20B, inset). On 8 cells it was actually possible to carry out the test protocol in ACSF first and then to repeat it in TTX solution. The potentiation of CCh response by SPME was maintained in TTX only in half of the cells tested.

The described potentiating effect of SPME was dose dependent (see Fig. 23 for data on the same cell; n=3) and probably mediated via NK_1 receptors, as it was blocked reversibly by the specific NK_1 antagonist SR1403333 (n=5, see Fig.21B, Fig. 22B).

In summary then, these observations suggest that SPME facilitated the membrane excitation induced by CCh and largely enhanced the network ability to generate rhythmic bursts as well as low amplitude, repetitive oscillations. At the same time the extent of the depression by CCh of electrically evoked synaptic events, a phenomenon presumably due to the drug ability to block transmitter release presynaptically (Segal, 1989; Marchi and Raiteri, 1989), remained the same in the presence of SPME.

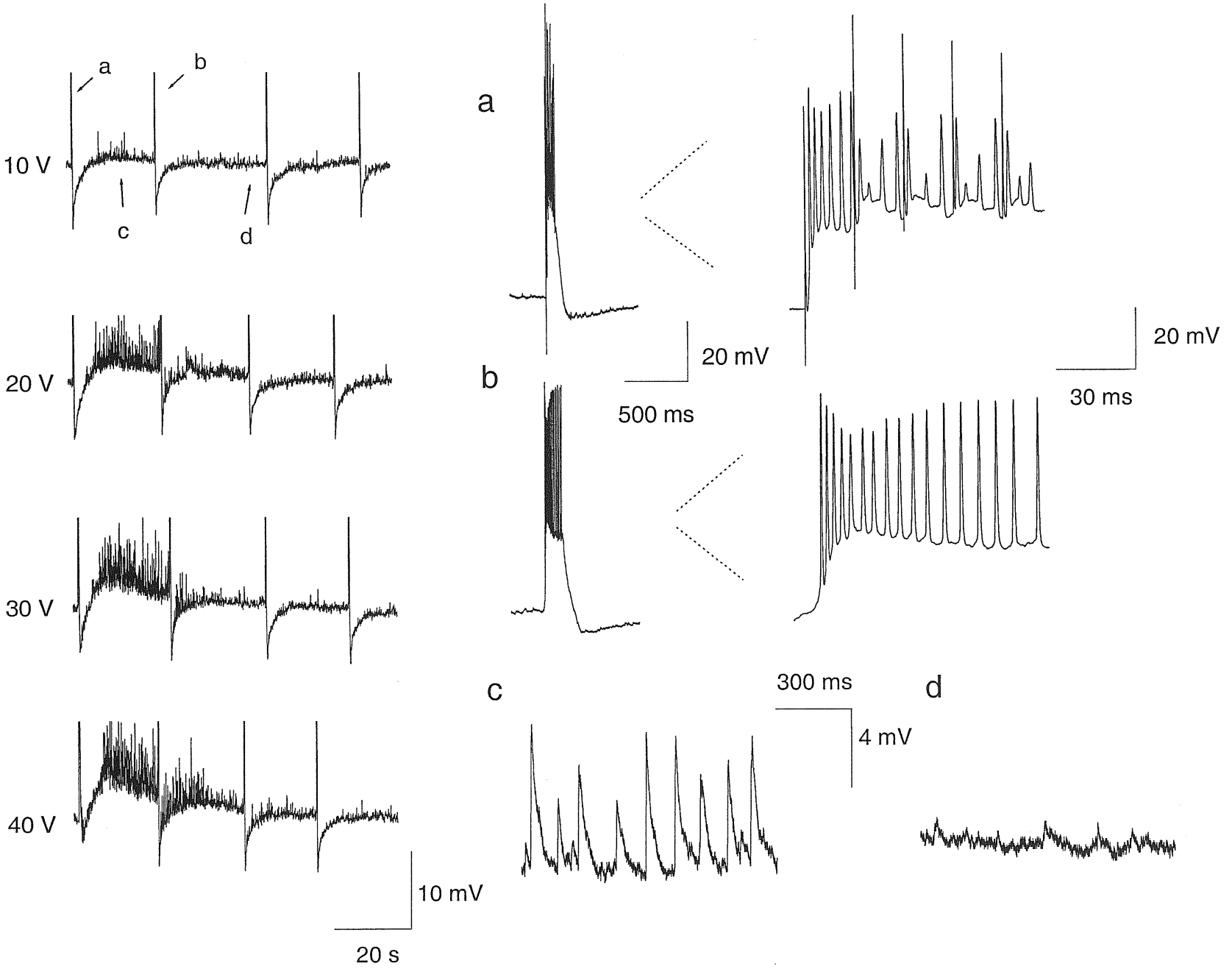

Fig. 24 page 84

Fig. 24. Characteristics of evoked slow cholinergic EPSP. Typical components of sEPSP consist of slow depolarization that follows stimulus artefact (see upward defflections at the start of the trace), increased spontaneous excitatory events on top of depolarization (c, compare to control background activity in d) and spontaneous synchronous bursts (b, enlarged in the insert). Note stimulus intensity dependence of all sEPSP elements. Fast EPSPs with irregular spikes on top of the depolarization and AHP that followed stimulus artefact are expanded in the inset a) for comparison with spontaneous bursts in b). All traces are from the same cell recorded in hydrophonium chloride solution (3 μM) with KCl-filled electrode. RMP=- 63 mV.

4.8. Modulation of endogenous cholinergic responses by SPME

In order to evaluate a possible physiological relevance of this modulating effect of SPME to the hippocampal cholinergic pathways we studied evoked slow cholinergic EPSPs in the presence of this neuropeptide.

Slow cholinergic EPSPs (sEPSPs) were induced using a classical protocol (Cole and Nicoll, 1984) by electrical stimulation of cholinergic fibres at s. oriens/alveus border (see Methods for parameters) in slices pre-treated at least for 20 min with the acethylcholinesterase blocker edrophonium chloride (3 μM). The distance between the stimulating electrode and the recording one was about 1 mm.

Cholinergic sEPSPs recorded with KCl electrodes were evident as a stimulus intensity- and voltage-dependent depolarization that lasted for 10-80 s (see Fig. 24, left panel). A characteristic feature of these sEPSP was the intense oscillatory activity which accompanied the sEPSP peak depolarization. This is exemplified in the record of Fig. 24c, where the trace is shown on a faster time base (compare it with baseline in d, before the stimulus train). In addition to this enhanced synaptic activity, 42% of cells tested (9/21) also displayed spontaneous bursting, characterised by a large depolarization (often exceeding 20 mV) superimposed with a highly regular barrage of action potentials (see example in Fig. 24b, which is shown at a medium or very fast time base to reveal its components) followed by AHP. The slow and repetitive bursting activity was different from the early cluster of action potentials (followed by an AHP) generated by the short latency fast EPSP as initial response to the stimulus train (see example in Fig. 24a). In fact, during these early EPSPs spike firing was irregular and unaffected by atropine.

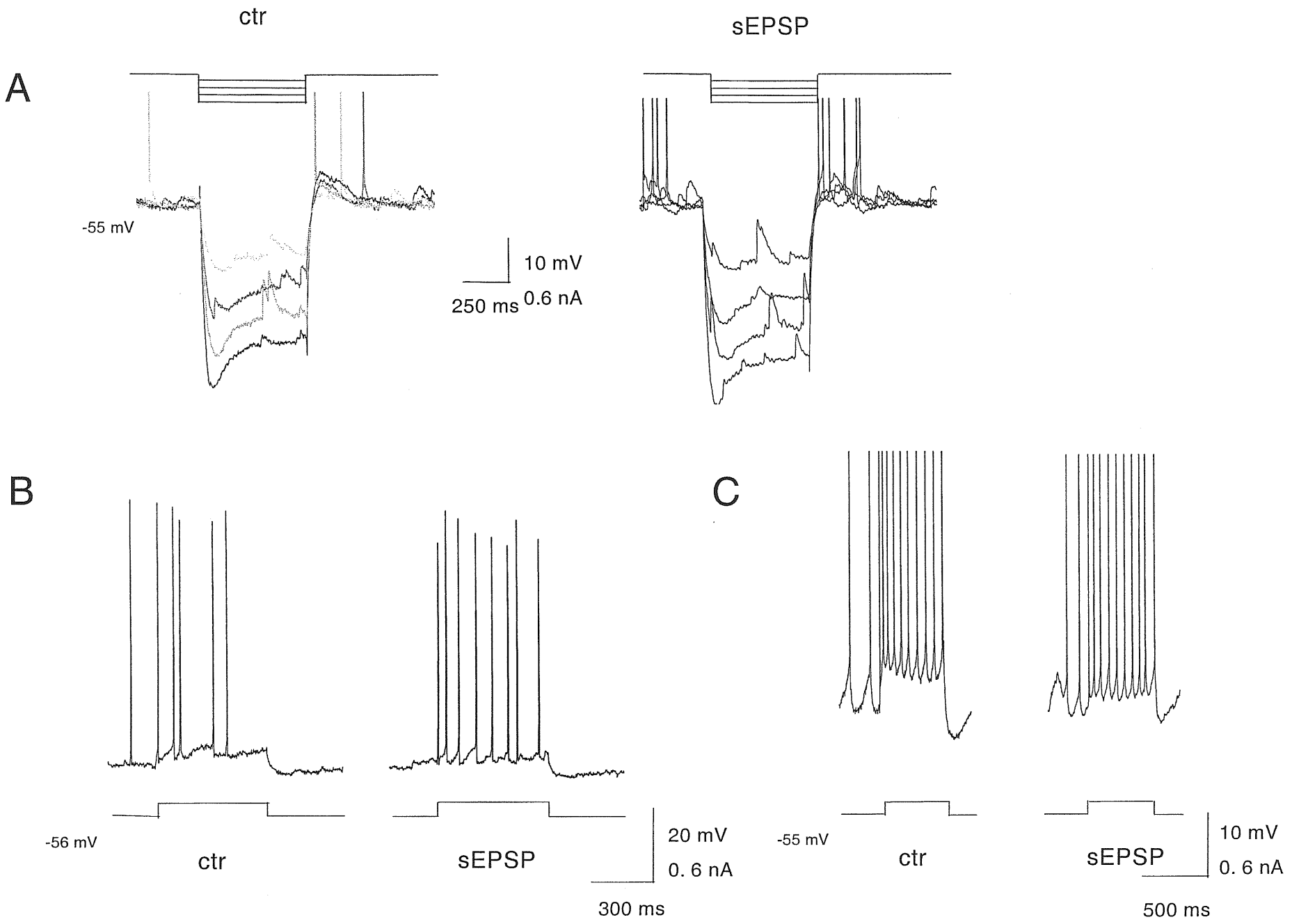

Fig. 25 page 86

Fig. 25. Membrane properties during the evoked cholinergic sEPSP. A. Voltage responses to negative current steps applied in control (left panel) and during s. alveus/oriens stimulation (right panel). Note an increased input resistance during sEPSP. B. Block of accommodation during sEPSP. Note that in response to the same step of injected current (+0.1 nA) cell instead of 5 spikes (control, left trace) fired 8 spikes (right trace). C. Reduction in AHP during evoked cholinergic response. Note that the same train of 10 spikes during the sEPSP (right trace) generated smaller AHP than in control (left trace). All recordings are obtained with a KCl electrode in hydrophonium chloride solution (3 μM).B and C are from the same cell.

Slow depolarization, increased oscillatory activity and synchronous large discharges (if present) were blocked by atropine (10 μM, bottom trace in Fig. 26A, n=7), indicating that they were all produced by stimulation of cholinergic fibres.

During sEPSP depolarization a small increase in cell input resistance was detected in comparison with the situation when the cell was manually clamped at the same membrane potential at rest (see Fig. 25A; 18±9%, n=7). Other typical electrophysiological characteristics of the cholinergic sEPSP such as decrease in spike accommodation and reversible reduction in the AHP were also observed when a positive step of current was applied during the s EPSP (see Fig. 25B and C).

For experiments with SPME cells were maintained at around -60 mV (RMP=60±4 mV) by continuous positive current injection, since the probability of the generating a sEPSP, as for any other cholinergic response, is highly voltage-dependent (see Fig. 26B for an example with CCh application). The stimulus parameters were adjusted to produce a moderate sEPSP response with a peak depolarization equal to 1/2 -1/3 of the maximum value. An example is in Fig. 26A (top trace) in which in control conditions a stimulus of 20 V elicited a small 2.5 mV sEPSP. The next trace shows that in the presence of SPME the cholinergic response induced by the same stimulus was increased threefold (8 mV). Background noise was increased and prolonged in the duration. Among 18 cells tested with this protocol 16 showed a potentiation of the sEPSP in the presence of 0.1 μM SPME, evident as an increase of the amplitude of depolarization (5±1mV versus 3±1, $P<0.01$) and augmentation of the background noise during the response. The remaining two cells showed only an increase in synchronous bursting. Application of SR140333 (4 μM) for 20 min did not alter the amplitude of sEPSP, but in the presence of this NK_1

antagonist SPME was not able to produce the potentiation of sEPSP seen before (Fig. 26B, n=3).

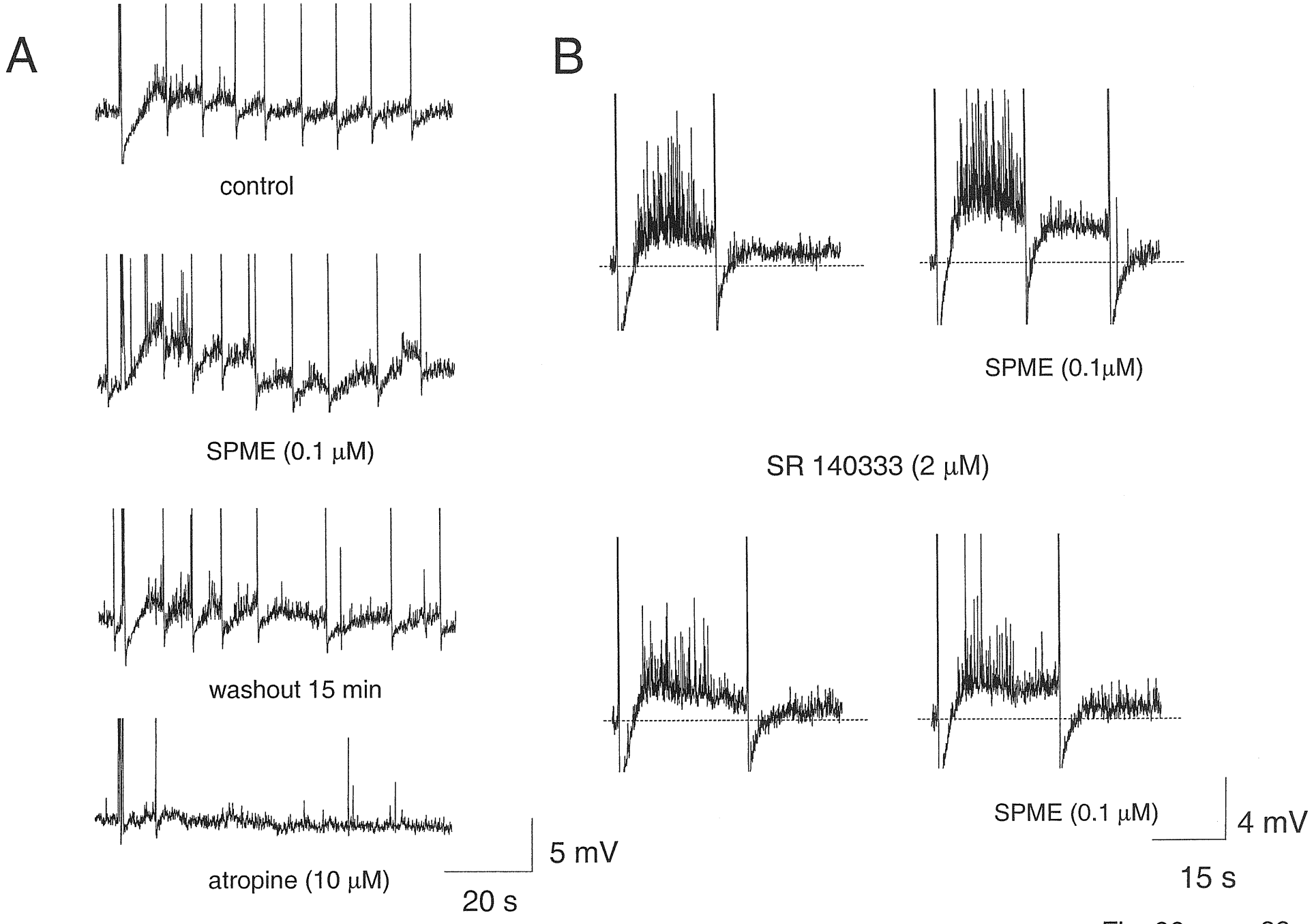

Fig. 26 page 89

Fig. 26. SPME potentiated sEPSPs via activation of NK_1 receptors. A. SPME (0.1 μM) reversibly increased depolarization and spontaneous activity of the evoked sEPSP. All components of the sEPSP are blocked in the presence of 10 μM atropine. RMP=-55 mV. B. Potentiation of cholinergic sEPSP by 0.1 μM SPME is absent after the cell is exposed for 20 min to 4 μM SR140333 (cell different from A, RMP=-60 mV). Although the amplitude of the sEPSP during NK_1 receptor antagonist application is similar to the one in control, a slight reduction in background noise on top of the depolarisation is evident. The latter effect might be caused by antagonism of tonic activity of endogenous SP. All recordings done in edrophonium chloride solution (3 μM) with KCl electrode.

V. DISCUSSION

The principal finding of this study is the novel demonstration of a complex modulatory action by the endogenous occurring peptide SP (or its synthetic analogue SPME) on fast (GABA and glutamate) and slow (cholinergic) synaptic transmission recorded from CA1 pyramidal neurones in the mouse hippocampal slice preparation. Electrically-evoked synaptic responses, both excitatory and inhibitory, were usually depressed while the frequency of spontaneous GABAergic events was increased. Pre-treatment with the neuropeptide potentiated the endogenous cholinergic response as well as the one elicited by CCh application.

5.1. Effect of SPME on basic membrane properties and excitability of the principal neurones:

SP and its analogues are known to elicit strong excitatory effect on various principal neurones of the CNS (Otsuka and Yoshioka, 1990), amongst which are spinal cord motoneurones (Fisher and Nistri, 1993), dorsal horn neurones (Murase and Randic, 1984), basal forebrain (Stanfield *et al.*, 1985) or striatal (Bell *et al.*, 1998) cholinergic neurones, locus coeruleus neurones (Shen and North, 1992), the anterior cingulate cortex neurones (Jones and Olpe, 1985), nucleus of the solitary tract and dorsal motor nucleus of the vagus neurones (Maubach and Jones, 1997). In these cases the electrophysiological effect of SP is often a prominent slow depolarization (usually exceeding 10 mV), associated with increase or decrease in cell input resistance: these phenomena have been ascribed to a direct effect of the neuropeptide on several ionic conductances, including a decrease in K^+ conductance due to block of the fast inward rectifier (Stanfield *et al.*, 1985; Shen and North, 1992; Bell *et al.*, 1998), or inhibition of the Ca^+-activated K^+ current or suppression of the leak current carried through K^+ channels (Fisher and Nistri,

1993) and/or increase in some non-selective cationic conductances (Murase *et al.*, 1989; Shen and North, 1992) perhaps activated by Ca^{2+} (Fisher and Nistri, 1993).

In contrast to these data, in our study on pyramidal cells of the mouse hippocampus SPME produced only small changes in excitability (shallow depolarization, alterations in spike rheobase, latency, threshold, AHP and in the spike accommodation). Intracellularly recorded effects of SP have been previously found only very occasionally on rat CA1 neurones (Dodd and Kelly, 1981), although those experiments relied on pressure applications of the peptide which might have been delivered in insufficient amounts (for relatively short periods) to act on pyramidal cells. A slight effect of SP (small hyperpolarization with no change input resistance) on the principal neurones in the EC was also recently described by Maubach *et al.* (1998).

The very limited magnitude of these responses to SP and SPME did not allow a detailed characterisation of the mechanisms underlying them. Such modest changes in basic membrane properties and excitability produced by SPME in the hippocampus are in agreement with histochemical observations indicating that pyramidal cells lack SP receptors (Freund and Buzsaki, 1996). However, since the effect of SPME was still evident in TTX solution and hippocampal pyramidal cells are thought to be potential targets for SP-immunoreactive fibres (Sereth and Leranth, 1996), one can suspect that a small amount of NK receptors were located on the membrane of CA1 cells, although their number remains below the level detectable by standard histochemistry.

Since the slight direct effects of the peptide on CA1 pyramidal cells were unable to account for the large changes in synaptic transmission (recorded both extra- and intracellularly) these observations indicated a modulatory action of SPME rather than a

neurotransmitter role in this brain region. In fact, the peptide is known to enhance glutamatergic transmission in spinal cord (Smullen *et al.*, 1990) and to facilitate release of acetylcholine from the cerebral cortex (Sastry, 1995).

5.2. Depressant action by SP (or SPME) on field potentials

SP decreased the amplitude of field potentials evoked by electrical stimulation of *SR* fibres with a slowly developing action characterised by slow recovery and subsequent tachyphylaxis. The last phenomenon has been frequently observed for SP in neurones (Maggio, 1988) and is presumably due to transient internalization of its membrane receptors (Mantyh *et al.*, 1995). This feature was taken into account when the experimental protocol for SP applications was designed. The stable analogue of SP, SPME, which is known to be a selective agonist for NK_1 receptors (Regoli *et al.*, 1994), mimicked the depressant action of the native neuropeptide. Since the effects evoked by either SP or SPME were equally blocked by the NK_1 receptor antagonist SR 140333, it seems likely that the action of SP was mediated by NK_1 receptors. These receptors are known to be present in the hippocampus (Humpel and Saria, 1993). SPME was used for the majority of the present experiments because of its chemically stability, NK_1 receptor selectivity and apparent higher potency on synaptic transmission compared with SP. It is noteworthy that the maximum effect of SPME was found at a concentration of 0.1 μM, indicating a rather powerful action of this compound. No complete suppression of synaptic transmission was produced by SPME , as the effect saturated at about $1/3^{rd}$ reduction of the field potential.

The extracellular data, although intrinsically limited in their resolution, did provide several clues to the interpretation of the mode of action of SP on hippocampus. First, that the depressant action of the peptide required intact $GABA_A$ receptors. Second, that neither NMDA nor non-NMDA receptors were preferentially involved. Third, that the hippocampal thrisynaptic circuit was essential to observe this depressant effect in full, although a residual, small depressant action of SPME on the field potential recorded in the CA1 region persisted when the dental gyrus or CA3 area was removed. Thus, it seems likely that the synaptic depression took place at network level and that, at some stage, it comprised presynaptic modulation of endogenous glutamate release.

5.3. Modulation of evoked EPSPs and IPSPs: intracellular observations

Sharp intercellular recordings from the CA1 pyramidal region showed that on the majority of cells SPME depressed evoked glutamatergic EPSPs and GABAergic IPSPs by a comparatively similar degree.

The present tests with NMDA or AMPA applications in TTX solution suggested that the observed EPSP reduction was probably not due to a transient downregulation of glutamate receptor activity. Curiously, when the IPSP was studied in isolation, in the presence of kynurenic acid, the depression of this potential by SP was lost, implying that a glutamatergic drive was necessary for this effect. Maubach *et al.* (1998) actually described a small but significant increase in the amplitude of isolated both $GABA_A$ and $GABA_B$ IPSPs during application of SP.

Complementary studies on the pharmacologically isolated EPSPs during block of inhibitory synaptic transmission could not be completed because the sustained presence

of bicuculline led to development of convulsive activity and recording instability over the long term required for these experiments. However, the present extracellular experiments showed that the depression of the field potential, which is mainly made up by the glutamatergic EPSP, was absent in the presence of bicuculline.

5.4. Modulation of spontaneous synaptic potentials by SPME

Spontaneous GABAergic events (regardless of their polarity) were found to be consistently and reversibly increased in frequency (though not in amplitude) by SPME. This effect was again dependent on an intact glutamatergic drive since kynurenic acid or TTX strongly reduced or prevented such a frequency enhancement. Although those recordings obtained with K acetate electrodes should be interpreted with caution (since small events were probably comprised in the background noise and thus escaped detection) the fact that TTX curtailed the action of SPME on the frequency of spontaneous events indicates that our recording conditions could detect changes in event frequency (see for instance Hosokawa *et al.*, 1994), albeit perhaps restricted to a certain population of responses.

While intracellular recording from neurones with a complex distribution of synaptic inputs over a wide dendritic arborization makes them unsuitable for quantal analysis, it seems feasible to assume that the frequency of spontaneous events reflects impulse-dependent neurotransmitter release, which it turn is converted into basal release (miniature postsynaptic potentials) by TTX. The present data with TTX or kynurenic acid application suggest that SPME potently modulated GABA release only when the local circuit was pharmacologically intact. A complex circuit modification is thus envisaged to

account for this phenomenon. Analogous considerations showing that, following TTX application SPME did not change the frequency of miniature excitatory events, led us to propose a network site of action for SPME in reducing the EPSP frequency.

5.5. Cholinergic response in the hippocampus and its SPME modulation:

In the rodent hippocampus, as well as in most areas of the mammalian CNS, cholinergic responses are mediated mainly by muscarinic receptors (for review see Jerusalinsky *et al.*, 1997). Nicotinic receptors are supposed to modulate rather than mediate neuronal transmission in the brain (Jones and Yakel, 1997). In accordance with this notion, cholinergic effects, that were produced in our study either by exogenous application of CCh (potent agonist on both types of cholinergic receptor) or by ACh endogenously released during stimulation of cholinergic fibres, were completely abolished by the muscarinic antagonist atropine. The observed excitation by CCh of mouse pyramidal neurones was rather similar to the one reported in previous publications on rat hippocampal cells (Benardo and Prince, 1982; Cole and Nicoll, 1984; Pitler and Alger, 1992) and consisted in membrane depolarization (characteristically voltage-dependent) and input resistance decrease (or no change). In the present experiments, we frequently observed that CCh application or electrical stimulation of cholinergic fibres generated slow synchronised events. In the literature such events have been considered more closely related to epileptiform bursting (Williams and Kauer, 1997) rather than to the theta-rhythm observed *in vivo,* as earlier suggested by Bland *et al.* (1988). The main components of cholinergic action, namely depolarization and increase in spontaneous discharges, were presumably mediated by M1 type muscarinic receptors as they were

blocked by pirezepine. In a minority of cases application of CCh induced a small membrane hyperpolarization associated with a fall in input resistance; sometime this response occurred early during exposure to the cholinergic agonist to be later replaced by the more usual membrane depolarization. Previous experiments on rat dissociated hippocampal neurones have also reported a CCh evoked hyperpolarization (Wakamori *et al.*, 1993), probably mediated by a direct increase in membrane permeability to K^+ via activation of M3 muscarinic receptors. Finally, CCh also depressed the amplitude of electrically evoked EPSPs and IPSPs in mouse hippocampal neurones: this effect has previously been observed in the rat hippocampus and has been attributed to presynaptic modulation of transmitter release via M2 receptors (Marchi and Raiteri, 1989; Segal, 1989).

All components of the excitatory response elicited by CCh were potentiated by SPME in a dose-dependent manner. The large augmentation of the CCh induced depolarization was not associated with a large increase in resting input resistance of the recorded CA1 cells. This observation suggests that the SPME facilitation was not an epiphenomenon of a generalized increase in membrane resistance which made the cell more sensitive to postsynaptic activation. Indeed, responses to glutamate receptor agonists were not significantly potentiated by SPME. It seems likely then that SPME improved the coupling efficiency of muscarinic receptors to their effectors once they bound CCh (note that to avoid over-excitation of neurones we selected CCh doses which gave relatively small, submaximal responses).

CCh is known to act through a variety of muscarinic receptor subclasses in the brain (for review see McKinney, 1993). Virtually all the excitatory effects of CCh were enhanced

by SPME whereas the depression of electrically evoked transmitter release was left unchanged and the occasional membrane hyperpolarization was reduced and/or converted into depolarization. These observations indicate that not all effects of CCh were uniformly sensitive to SPME potentiation. However, the systematic increase in CCh excitatory responses may suggest that SPME acted indirectly through some common intracellular mechanisms generally important for the operation of certain classes of muscarinic receptor. Future studies based on the analysis of the changes in intracellular signal trafficking brought about by SPME should help to clarify this issue. In addition to the larger membrane depolarization produced by CCh, it was interesting to find out that repetitive oscillatory activity was also increased, indicating that the presence of SPME shifted the operation of the hippocampal network towards a more synchronous pattern of discharge. The oscillatory activity detected from mouse CA1 cells had considerable analogies with the one already described for the rat hippocampus (Pitier and Alger, 1992; Behrends and Bruggencate, 1993; MacVicar and Tse, 1989; Williams and Kauer, 1997). In particular, in the present study it was possible to identify *small amplitude and relative fast* membrane oscillations which were blocked by bicuculline (like comparable oscillatory responses recorded from rat neurones Pitier and Alger, 1992; Behrends and Bruggencate, 1993) and thus required intact GABA-mediated synaptic inhibition within the hippocampal network. This particular type of oscillation could be clearly detected only when the CCh induced membrane depolarization was quite small. When the CCh response comprised a substantial membrane depolarization, at the peak of the effect *large and slower bursts* emerged, typically lasting several seconds and with superimposed action potentials (often blocked by voltage dependent inactivation due to the sustained

membrane depolarization during each burst). Again, a similar type of response has been repeatedly found in rat hippocampal neurones (MacVicar and Tse, 1989; Williams and Kauer, 1997): in this case the origin of these bursts is thought to be focal excitation of CA3 cells by CCh which generates synchronous firing to recruit CA1 cells via glutamate receptor dependent transmission. Part of these bursts may also originate by facilitation of low threshold Ca^{2+} currents (see Strata, 1998). In the rat hippocampus these bursts are reported to be facilitated by block of GABA mediated inhibition. Finally, during the gradual return of membrane potential to rest, *intermediate amplitude, medium duration oscillations* (presumably sharing similar origin with large ones) appeared as also reported for rat neurones (MacVicar and Tse, 1989; Williams and Kauer, 1997). Their briefer duration and more variable size may have simply reflected a progressive exit of a certain number of hippocampal neurones from their collective synchronous operation as the local concentration of CCh was lowered.

All components of the excitatory response, elicited by CCh application, were enhanced by SPME in a dose-dependent manner. The neuropeptide could have induced this potentiation via interaction with its NK receptors, that are G-protein coupled, and activate intracellular kinases (Mantyh *et al.*, 1984; Nakajima *et al.*, 1989) to phosphorylate postsynaptic muscarinic receptors, or, alternatively, via direct allosteric modulation not involving a distinct peptide receptor system (this process is known to occur for example for nicotinic receptor modulation; Stafford *et al.*, 1994). Since the effect of SPME was blocked by the selective NK_1 receptor antagonist SR 140333, the first possibility seems to be more feasible. The potentiation of the response to CCh was present in ACSF and in about fifty percent of neurones re-tested in TTX solution. Probably the density of SP

receptors located on the postsynaptic membrane of pyramidal cells is very low as already mentioned, although even a small number of those receptors could enhance the muscarinic ACh-mediated response. In this case, one might speculate that despite the low receptor density there was considerable amplification of the signal (after receptor activation) by intracellular second messengers. In contrast, a direct effect of SPME via NK_1 receptors on the AMPA or NMDA receptors was not found in our experiments, whereas for example in dentate gyrus granule cells a modulatory NK_1 mediated effect of SP on NMDA receptors has been described (Lieberman and Mody, 1998). Unlike glutamate receptors, cholinergic receptors could perhaps be more sensitive to or closely coupled with NK receptors. As the SPME enhancing action was notably reduced in TTX, there is no doubt that the main mechanism of the phenomenon originates at network level.

The sEPSP, which is believed to be due to release of endogenous ACh mainly from septohippocampal fibres (Frotscher and Leranth, 1985), was also largely potentiated by SPME. Note that in the conventional, transversely cut hippocampal slice preparation the cell bodies of the cholinergic neurones responsible for the sEPSP have been severed: hence, afferent cholinergic fibres can be electrically activated but exhibit no significant spontaneous, ongoing activity. This feature makes it difficult to apply traditional methods of quantal analysis to understand the locus of action for any observed changes in synaptic transmission since no spontaneous cholinergic events were evident in resting conditions and atropine did not apparently change spontaneous synaptic activity in control solution. There is also a small number of local cholinergic interneurones which are mainly distributed through *SLM* and sparsely in *SR*, *StP* of the CA1 (Freund and Buzsaki, 1996) but they are unlikely to have contributed to the generation of sEPSPs in view of the location of the stimulating electrode at the border between *SO/alveus*. These

considerations make improbable that sEPSPs were facilitated by SPME because the peptide activated cholinergic cells presynaptically.

The experimental constraints of the present study do not allow a clear identification of the mechanism of action through which SPME enhanced cholinergic sEPSPs. It is however possible to formulate a few hypotheses. For example, SPME might have increased ACh release directly, perhaps by enhancing Ca^{2+} currents (see Murase *et al.*, 1989) or blocking some K^{+} currents (Stanfield *et al.*, 1985; Shen and North, 1992; Fisher and Nistri, 1993; Bell *et al.*, 1998) of cholinergic terminals. However, there is no evidence for NK receptors strategically localised on cholinergic terminals.

SPME might have enhanced the sEPSP via a network interaction with other transmitters. The most probable candidate for this role is GABA, particularly because the present study demonstrated that SPME facilitated spontaneous GABA release within the hippocampal network. In turn GABA is known to enhance release of ACh as shown in neurochemical experiments on rat hippocampal synaptosomes (Bonanno and Raiteri, 1987). The mechanism responsible for this effect of GABA remains uncertain although it has been shown to be blocked by GABA uptake inhibitors. In addition to a presynaptic facilitation of ACh release, the present data suggest that SPME might have also increased postsynaptic responsiveness of pyramidal cells to ACh since in a number of neurones bathed in TTX solution the postsynaptic action of the cholinergic agonist CCh was also increased. It is also interesting to point out that cholinergic transmission in the hippocampus can have profound modulatory effects which go beyond the immediate depolarization of pyramidal cells. In particular, local cholinergic activity has been suggested to greatly diminish GABA-mediated inhibition of pyramidal cells, thus causing disinhibition of GABAergic interneurones which could further raise pyramidal cell excitability (Krnjevic *et al.*, 1981), a phenomenon recently demonstrated by Toth *et al.* (1997).When this cholinergic activity was enhanced by SPME, the hippocampal network became more susceptible to bursting and generated synchronous discharges as indeed

observed in the present study in accordance with the spontaneous synchronous bursting evoked by cholinergic disinhibition (Toth *et al.*, 1997).

5.6. Network mechanisms subserving the modulatory effect of SPME

If an explanation for the mode of action of SPME is sought at network level, it may be useful to attempt discussing what cells might be involved and to outline a tentative wiring diagram to account for the various features of the action of SPME recorded from CA1 pyramidal cells. It should be first considered that the large majority of interneurones in the hippocampus are GABAergic (Freund and Buzsaki, 1996). In the CA1 region in particular, GABAergic interneurones are typically found in the *SO*, *StP*, *SR* (Buhl *et al.*, 1994; Freund and Buzsaki, 1996; Morin *et al.*, 1996 and references therein). Since *SO* cells make up the main spontaneous GABAergic input to CA1 pyramidal cells (Lacaille *et al.*, 1987; Lacaille, 1991) and in the present investigation the stimulating electrode was localized in the *SR* (or at the *SO*/alveus border), for sake of simplicity the role of GABAergic interneurones of the *StP* will not be further considered. *SO* cells, which receive a glutamatergic input from CA3 and CA1 pyramidal cell collaterals (Lacaille *et al.*, 1987; McBain *et al.*, 1994), are spontaneously-active and comprise distinct cell types which project to CA1 and CA3 pyramidal cells (Sik *et al.*, 1994), and to *SR* where they are suggested to establish contacts with the local GABAergic interneurones (Bernard and Wheal, 1994) even if such a direct connection could not be found when recording from a small sample of these cells (McBain *et al.*, 1994). *SR* GABAergic interneurones are not spontaneously active, receive a glutamatergic input from the Schaffer collaterals (Kunkel *et al.*, 1988) and project to CA1 pyramidal cells to mediate feed forward inhibition (Lacaille *et al.*, 1987; Ouardouz and Lacaille, 1997). Although most of these studies were carried out on rat hippocampal tissue, it should be noted that similar organization and function of the *SO* interneurones are also found in the mouse (Yanovsky *et al.*, 1997).

Fig. 27 presents an idealized diagram which attempts to provide a unitary hypothesis to

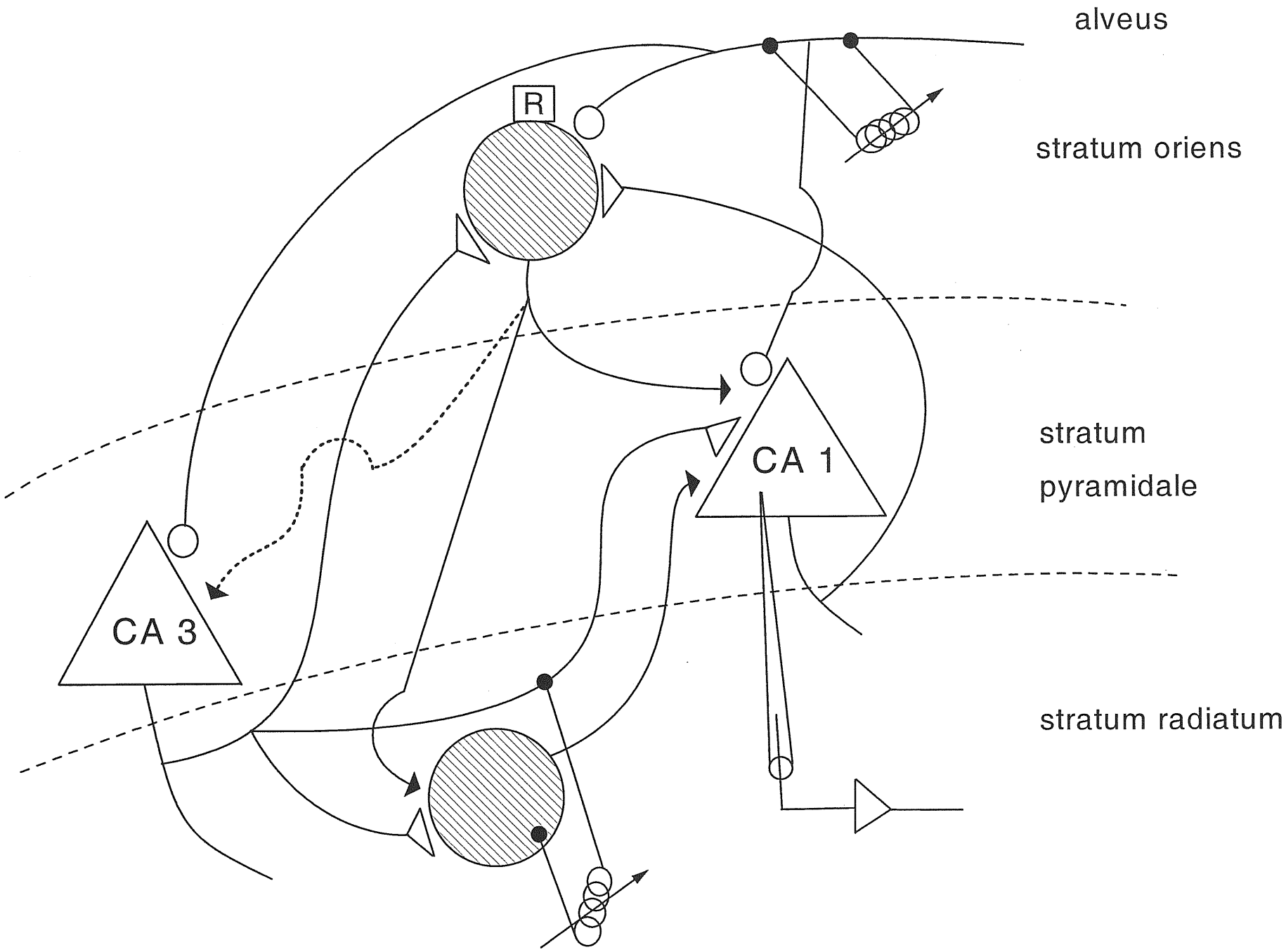

Fig. 27 page 102

Fig. 27. Idealized diagram of minimal network connections used to account for the action of SPME on synaptic transmission in the CA1 area as detected by intracellularly recording from a CA1 pyramidal neurone. The drawing is not scaled to cell size and does not depict dendritic arborization for sake of simplicity. GABAergic interneurones are identified by their shaded cell body and can be found in the *SO* (top) or in the *SR* (bottom): their projections are indicated by arrows. Only the *SO* interneurone is supposed to possess NK_1 receptors (labelled as boxed R) which, once activated by SP, are presumed to excite this cell. *SO* interneurone sends inhibitory inputs to CA1 and CA3 pyramidal cells (dashed line represent the projection established *in vivo*) and to *SR* interneurone. Pyramidal CA1 cell would then receive enhanced inhibitory signals from *SO* cell (detected as increased frequency of GABAergic events) as well as reduced inhibition from *SR* interneurone (detected as reduced size of electrically evoked IPSP; see symbol for stimulating electrodes). The *SO* interneurone also inhibits the excitation from CA3 to CA1 cell via the Schaffer collaterals leading to decrease in the electrically evoked EPSP. Normally *SO* interneurone is spontaneously active and receives excitatory inputs from CA3 and CA1 pyramidal cells: such inputs may be facilitated by SPME. Cholinergic fibres, which projections are indicated by open circles, impinge on pyramidal cells (CA1,CA3) and *SO* interneurone. Once stimulated at *SO*/alveus border (note symbol for stimulating electrode on top) cholinergic fibres bring about an excitation of pyramidal cells (seen as enhanced excitability, slow synchronous bursts and membrane potential oscillations at the level of the CA1 pyramidal cell) and activate *SO* interneurone in a manner already discussed for SPME (seen as an increase of biccuculline sensitive fast oscillations and decrease of the electrically evoked IPSPs). Note that SPME activation of *SO* interneurone can produce disinhibition of the CA1 pyramidal cell from *SR* interneurone, and might in part account for the neuropeptide potentiation of the excitatory effect of ACh.

account for the multifarious and apparently contrasting effects of SPME on synaptic transmission. Most SP receptors are found at the level of GABAergic cells in the *SO* interneurones (Acsady *et al.*, 1996). In analogy with the general excitatory action of SP on central neurones (Otsuka and Yoshioka, 1993) it seems likely that the peptide might similarly facilitate the activity of *SO* interneurones.

In the present study evidence in favour of such increased interneuronal activity comes from the observation that spontaneous GABAergic activity measured at the level of pyramidal neurones was greatly enhanced by SPME.

The fact that extracellular field potentials generated by stimulation of Schaffer collaterals were depressed by SP or SPME application is also in keeping with the proposed activation of non-principal cells. In fact, SP, by facilitating release of the inhibitory transmitter onto the principal cells, would have decreased the amount of synaptic glutamate delivered to principal cells. The indirect origin of the action of SP on glutamatergic transmission is confirmed by the observation that there was no preferential reduction of a certain glutamatergic component of the field potential. Furthermore, glutamatergic transmission became insensitive to SP in the presence of bicuculline. Several other findings, obtained with the higher resolution intracellular recording, are fully compatible with the view that SP modulated glutamatergic transmission via enhanced GABAergic transmission. In particular, one can list the attenuation of electrically evoked EPSPs, the lack of any modulatory effect of SPME on AMPA or NMDA-induced responses in TTX solution and the inhibition of spontaneous glutamatergic events.

More complex appears any attempt to explain the decrease in IPSPs evoked by Schaffer collaterals stimulation during SPME application. Curiously, the IPSP was depressed only when it followed the EPSP whereas the pharmacologically isolated monosynaptic IPSP was unaffected (the latter finding appears to rule out any direct action of the peptide on these GABA releasing neurones or their GABA receptors). Note that the insensitivity of

the monosynaptic response to SPME is not surprising because there are few SP receptors in this CA1 area. (Acsady et al., 1996). The scheme of Fig. 27 can however provide a network based explanation for these phenomena. In fact, *SO* interneurones activated by SPME should inhibit GABAergic interneurones located in *SR,* making them less excitable by electrical stimuli applied to the same area.

The exact mechanism responsible for the upregulation of non-principal cell (presumably, *SO* interneuron) activity has not been addressed in this study and has not been described in the literature. Recently Maubach and Jones (1998) have demonstrated that application of SP to the medial entorhinal cortex produces substantial depolarization of putative inhibitory interneurones while it has little effect on principal cells from the same brain area. Like for brainstem neurones (Shen and North, 1992) or spinal motoneurones (Fisher and Nistri, 1993), one may envisage that on interneurones SPME increased excitability by blocking certain K^+ currents and by activating non-selective cationic currents. Another possibility is that SPME facilitated the action of glutamate via a metabolic effect (Urban *et al* 1994) on *SO* interneurones. This latter scenario is compatible with the present observation that in kynurenic acid solution SPME lost most of its ability to facilitate spontaneous GABAergic events and, in particular, that the monosynaptic IPSP from the *SR* region was unchanged by SP. If the latter observation (obtained in kynurenic acid solution) merely involved a disynaptic, purely GABAergic process (enhanced released of GABA from *SO* cells with inhibition of *SR* cells), one would expect to find the *SR* cells less excitable and thus to detect their IPSPs smaller. Viceversa, if SP potentiated the action of glutamate to activate *SO* neurones, block of glutamate receptors would have impaired the action of the peptide. The importance of a glutamatergic input to *SO* cells is also shown by the "mini-slice" experiments in which ablation of CA3 or dentate gyrus region (which contribute the main glutamatergic inputs) substantially reduced the depressant action of SPME. It should be noted that the interpretation of the present data has been simplified by leaving out the possible role of mGluRs. Future studies are

required to investigate their potential role by using appropriate agonists and antagonists. It has been considered that *SO* GABAergic interneurones act as "gatekeepers" of CA1 pyramidal cell activity (McBain *et al.*, 1994). In accordance with this view the proposed action of SP at the level of the *SO* would introduce a change in gain setting of the inhibitory network which can profoundly affect the operation and thus the output of pyramidal cells.

Within the framework of this simplified scheme the action of the cholinergic pathways would be unmasked only when these fibres are directly activated by electrical stimulation in view of the absence of cholinergic cell bodies in the slice preparation. The cholinergic projection to the hippocampus is diffuse as there are few areas devoid of cholinergic innervation (Froscher and Leranth, 1985). It is also worth noting that the electrical stimuli required to induce a sEPSP mediated by ACh were strong and repeated. In these circumstances it is likely that there was a complex activation of the hippocampal network. In particular, part of the cholinergic fibres directly impinge upon CA1 (and CA3) pyramidal neurones which through recurrent collaterals will then activate GABAergic cells as well as adjacent pyramidal cells (see Fig. 27). This will result in widespread, patterned excitation as indeed was often observed during the late part of the sEPSP. The cholinergic projection to GABAergic interneurones will perhaps activate these cells in a fashion similar to the presumed action of SPME: this is compatible with the observation of low amplitude oscillatory activity which was sensitive to bicuculline block.

SPME might have enhanced the action of ACh either via upregulation of muscarinic receptor activity in pyramidal cells and interneurones (see experiments on CCh potentiation in TTX solution) or via facilitation of local GABA release which in turn could have stimulated the release of endogenous ACh (Bonanno and Raiteri 1987) or a combination of these two processes. A difficult question is to define how much SPME potentiated the cholinergic EPSP and how much it simply added an excitatory signal to the one generated by ACh on the same GABAergic target cells. The answer to this

question can only come from recording the responses of single GABAergic interneurones to SPME before and during activation of cholinergic fibres. It is however interesting to observe that on pyramidal cells SPME could enhance cholinoceptor mediated excitation at postsynaptic level even if the peptide produced minimal changes in membrane potential or input resistance. This finding would be compatible with an effective modulatory role of the peptide which could amplify the intensity and duration of effects induced by ACh on hippocampal cells. In view of the proposed role of the cholinergic system in learning and memory (Dutar *et al.*, 1995), it would be tempting to speculate that the reported ability of SP to facilitate learning in the rat (Huston and Hasenohrl, 1995; Sprick *et al.*, 1996) might at least in part to be due to enhancement of cholinergic synaptic transmission in the hippocampus.

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