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AN ELECTROPHYSIOLOGICAL STUDY OF THE EFFECTS OF THYROTROPIN RELEASING HORMONE (TRH) ON CA1 PYRAMIDAL NEURONS OF THE RAT HIPPOCAMPAL SLICE PREPARATION

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ABSTRACT

The electrophysiological action of TRH, a neuropeptide largely present in the CNS, was studied in a thick slice preparation of the rat hippocampus with intracellular techniques. Bath applications of TRH (5-20μM) did not affect either the input resistance or the resting potential of pyramidal cells (n=76). When a train of spikes elicited activation of Ca^{2+}dependent K^+ conductances resulting into an afterhyperpolarization (AHP), the AHP was decreased in amplitude by TRH which left unchanged the number of spikes and the interspike intervals. A voltage clamp study of high threshold voltage activated Ca^{2+} (HVA) conductances showed no effect of the neuropeptide on these currents measured at steady state.

Excitatory postsynaptic potentials (EPSPs), mediated both by NMDA and AMPA receptors, evoked after extracellular stimulation of the Schaffer collateral fibers were pharmacologically isolated. In TRH a transient and cospicuous potentiation of NMDA receptor mediated EPSPs was observed. The effect was specific since it did not affect AMPA receptor mediated responses. Bath application of NMDA (10μM) or AMPA (5μM) was tested on the same neurons. Unlike AMPA-induced responses, responses evoked by bath-applied NMDA were also transiently enhanced. A similar specific modulation was shown when the same protocol was applied in the presence of 1μM TTX, which suggests a very likely postsynaptic site of action of TRH. GABA mediated inhibitory postsynaptic potentials were evoked after stimulation of different regions of the hippocampus (i.e. *stratum lacunosum moleculare*, LM and *stratum pyramidale*, SP). TRH was decreasing the peak amplitude of $GABA_A$ receptor mediated responses regardless of the site of stimulation. On the other hand, $GABA_B$ receptor mediated responses were diminished only when evoked from the SP. Bath applications of isoguvacine and baclofen (10μM), $GABA_A$ and $GABA_B$ agonists respectively, elicited responses unaffected by TRH. These findings suggest a site of action of TRH which involves the presynaptic circuit. Finally, long and short term potentiation (STP/LTP) was induced in control condition (10μM picrotoxin) and after TRH delivery. TRH blocked the induction of the potentiation. In conclusion, TRH enhanced the excitability of pyramidal neurons by a combination of enhancement of excitatory transmission and depression of inhibitory one. In case of strong stimulation (i.e. STP/LTP) it tends to occlude further potentiation, suggesting its role of "damper" in the hippocampus.

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INTRODUCTION

1. THYROTROPIN RELEASING HORMONE (TRH)

1.1 PRESENCE AND LOCALIZATION OF TRH IN THE CNS

Thyrotropin releasing hormone, (TRH), is a weakly basic tripeptide (pGlu-His-Pro-NH_2) (BØler et al., 1969; O'Leary and O'Connor, 1995) (Fig. 1) produced by enzymic cleavage from a high molecular weight precursor containing five copies of the peptide itself (Segerson et al.,1987). It is principally synthetized in the hypothalamic cell bodies, transported to their axons terminals and then secreted into the portal vessel system through which TRH reaches its target cells in the anterior pituitary gland, where it induces release of prolactin followed by that of thyrotropin (TSH) (Jacobs et al., 1971). Nevertheless, the endocrine action of TRH appeared to be restricted not only to prolactin and TSH production but also to other hormones, e.g. growth hormone (Scanlon et al., 1983). Once released it is rapidly degraded by enzimatic cleavage (Griffiths, 1976) into two metabolites: acid-TRH and histidylproline diketopiperazine [cyclo(His-Pro)] (Griffiths et al., 1983), the latter has many effects on the brain including stimulation of the cortical neuron activity (Stone et al., 1991). However up to now no specific binding sites have been discovered for these TRH metabolites.

Recently, much interest was focused on the extrahypothalamic functions of TRH rather than its endocrine role. More than two thirds of the brain content of TRH are localized outside the hypothalamus. Quantitative autoradiographic techniques (Sharif,1989) have established that TRH receptor binding sites are widely distributed in the CNS (see Fig. 2), both in the spinal cord and in many regions of the brain particularly the amygdaloid complex and the hippocampal formation (Sharif,1989). Further studies using molecular biology techniques have obtained very similar

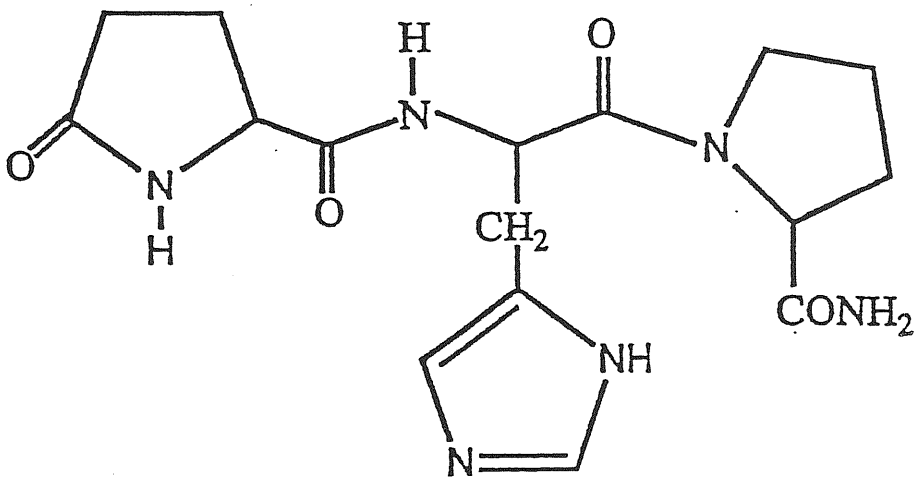


Fig. 1 Schematic figure of Thyrotropin Releasing Hormone (TRH) (O'Leary and O'Connor).

Tissue	Rat Brain		Mouse Brain	
	K_d	B_{max}	K_d	B_{max}
Retina[b]	5.4 ± 0.6	301 ± 38	6.3 ± 0.4	65 ± 3
Amygdala[b]	3.1 ± 0.5	153 ± 26	5.1 ± 0.3	222 ± 27
Pituitary[b]	2.2 ± 0.3	90 ± 4	4.3 ± 0.2	26 ± 2
Hypothalamus	5.6 ± 0.2	85 ± 8	4.6 ± 0.1	78 ± 16
Hippocampus	3.6 ± 0.5	48 ± 11	6.1 ± 0.3	61 ± 8
Spinal cord	3.6 ± 0.8	45 ± 11	4.7 ± 0.7	54 ± 8
Pons/medulla	4.2 ± 1.3	43 ± 11	6.0 ± 0.6	62 ± 8
Cortex	4.6 ± 0.8	40 ± 2	4.6 ± 0.6	53 ± 8
Septum	2.5 ± 0.3	39 ± 6	ND	ND
Olfactory bulb[b]	3.2 ± 0.6	39 ± 5	6.6 ± 0.7	82 ± 8
N. accumbens	3.5 ± 0.3	37 ± 1	ND	ND
Cerebellum	3.1 ± 1.1	19 ± 5	7.9 ± 1.0	40 ± 10

[a] The data are means ±SEM from 2–4 animals of each species; see Sharif and Burt[100] and Sharif *et al.*[109] for more details. K_d = nM; B_{max} = fmol/mg of protein; ND = not determined.
[b] Region showing species difference.

Fig. 2 Equilibrium binding parameters for TRH receptors in rat and mouse CNS (Sharif, 1989).

results concerning the regional distribution of TRH receptor messenger ribonucleic acid (mRNA) in the brain (Kaji et al.,1993). Data showing a cellular colocalization of the peptide with various neurotransmitters (Arvidsson et al., 1990) including GABA (Fleming and Todd, 1994) suggest a possible neuromodulatory role of this peptide. TRH administration induces a wide range of clinical effects, including a general excitatory action which leads to analeptic effects on general depression phenomena as happening during hibernation and drug administration, e.g. barbiturates and ethanol (Breese et al., 1975; Stanton et al., 1980). In the same direction in spinal motoneurons TRH increases the electrical activity and acts as a trophic factor during the development (Engel et al.,1983) or after injuries (Faden et al.,1983). On the other hand TRH can also limit the effects of neurodegenerative disease, such as Alzheimer's, or epileptic seizures (Albert et al., 1993; Renming et al., 1992). In summary the effect of this drug leads to an overall stabilization shifting "pathological" level of activity (starting from depression ending to overexcitation) to more "physiological" conditions.

1.2 TRH RECEPTORS AND INTRACELLULAR PATHWAYS

The TRH receptor molecule was shown to be a protein made by about 400 aminoacids, both in humans and rodents (Matre et al., 1993; Sellar et al., 1993). A comparison between the aminoacid sequences of the human/mouse/rat TRH receptor, indicates high homology, except for the -COOH terminal tail (Morrison et al., 1994). TRH binds stereospecifically to its receptors with very high affinity (Burt and Snyder, 1975) and is actively cleaved by enzymes (O'Cuinn et al., 1990). Unfortunately since no TRH antagonists are currently available, the possibility that the TRH effect is exerted through activation of other receptors, different from the above mentioned, can not be completely ruled out.

In pituitary cells TRH receptors have been found to belong to the large class of receptors which acts through binding and activation of guanosine 5'-triphosphate (GTP) binding proteins, the

socalled G-proteins coupled receptors (Boerge et al., 1991). TRH receptor is an intrinsic membrane protein which spans the membrane in seven regions and posesses an hydrophobic pocket laying in the center of the seven transmembrane regions acting as binding site (Perlman et al., 1994). Even if it has been reported that TRH receptors in the pituitary and in the brain have similar binding and biochemical properties (Sharif, 1989), it still remains controversial whether the actions of TRH as a hormone-releasing factor or as a neuropeptide are expressed in the same way.

A large body of evidence confirms that in the pituitary cells activation of TRH receptors is coupled with elevation in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. This effect is consequent to TRH binding to the G-protein coupled receptor, which catalyzes the hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP_2) by phospholipase C (PLC) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Gershengorn, 1986).

IP_3 and DAG thus constitute the main second messangers produced by TRH. IP_3 moves from a perimembrane region to an inner cytosolic compartment where it binds to its receptor located in the endoplasmic reticulum and causes an efflux of Ca^{2+} from intracellular stores leading to increase in $[Ca^{2+}]_i$. On the other hand, DAG activates protein kinases C (PKC) which phosphorylates various substrates. Both the rise in $[Ca^{2+}]_i$ and in PKC activity could lead to modulation of receptor channels, including those for excitatory and inhibitory aminoacids (Gerber et al., 1989; Leidenheimer et al., 1992).

1.3 ACTION OF TRH IN VARIOUS AREAS OF CNS

Because of the large body of clinical evidence showing the influence of TRH on development or injury, the effects of this neuropeptide were thoroughly studied on motoneurons. On spinal or hypoglossal motoneurons TRH application causes a clear excitatory action accompained by a depolarization and a subsequent increase in spontaneous firing (Bayliss et al., 1992; Nistri et al.,

1990; Rekling, 1990). In both cases these effects are found to be directly exerted at the level of the motoneuron membrane and involve a decrease in K^+ conductances, including one active at rest and partly sensitive to Ba^{2+} (Bayliss et al., 1992; Fisher and Nistri, 1993).

In the forebrain TRH also affects K^+ conductances but in a more complex way. On the majority of septal neurons TRH increases the amplitude of voltage dependent K^+ conductances, nevertheless there is a smaller population of these cells responding with a decrease in the same currents (Toledo-Aral et al., 1993). Furthermore in all cells application of the peptide increased $[Ca^{2+}]_i$ (Toledo-Aral et al., 1993).

1.4 NEUROMODULATORY ACTIONS OF TRH

Like many other neuropeptides TRH also exerts a modulatory role on neuronal activity. Modulation can be achieved in various ways: for example increasing input resistance/lowering spike threshold or modulating neurotransmitter action. Physiologically, modulation of neurotransmitters can be achieved by increasing either the amount of neurotransmitter released and/or its postsynaptic effect. In several areas of the brain dopamine (Kerwin, 1979) or acetylcholine (Giovannini et al., 1991) release is found to be enhanced by TRH.

On cortical neurons TRH strongly increases firing frequency if coapplied with acetylcholine, even if the tripeptide itself does not alter the excitability of these cells (Yarbrough, 1976). In hypoglossal motoneurons TRH enhances NMDA mediated responses in a voltage dependent manner (Rekling, 1992), even if this effect seems to result from an alteration in an intrinsic membrane property, e.g. increase in input resistance. On the other hand, NMDA mediated evoked potentials are potentiated by TRH in neocortical neurons without any apparent change in the input resistance of the cells (Kasparov et al., 1994). From these data it can be observed that TRH dependent modulatory effects may be expressed through several effectors.

1.5 ACTION OF TRH ON THE HIPPOCAMPUS

In hippocampal slices Ballerini et al. (1994) have observed that TRH has no effect at resting membrane potential, while it decreases a K^+ conductance activated at depolarized potentials. On dissociated pyramidal neurons TRH induces two different phenomena: a transient outward current followed by a sustained inward current (Ebihara and Akaike, 1993). Analysis of their equilibrium potential and experiments with pharmacological blockers suggest that both conductances are K^+ mediated. Furthermore, depletion of IP_3 sensitive Ca^{2+} stores as well as PKC inhibitors suppress both currents suggesting the involvement of the IP_3 pathway. Since TRH reverses natural depression in the hippocampus (Stanton et al., 1980), memory dysfunctions and allegedly exerts a protective role during epileptic seazures, we wanted to test TRH on hippocampal neurons.

2. THE HIPPOCAMPUS: FUNCTION IN THE CNS AND INTERNAL ORGANIZATION

2.1 FUNCTION IN THE CNS

The hippocampus is a structure that is ontogenetically formed after folding of the cerebral cortex into the ventricle and then expanding in its *lumen* (Stensaas, 1968). This is the reason why the hippocampus has a close relation with other areas of the cortex and, more in general, why it plays the role of a relay which receives information and stores it temporarily before transferring it to relevant areas of the cerebral cortex (Zola-Morgan and Squire, 1990).

The two main regions which project onto the hippocampal formation are the entorhinal cortex (Jones et al., 1992) and the medial septum with the nucleus of the diagonal band of Broca (Dutar et al., 1995).

During the last decades the hippocampal structures have been object of intense and thorough study because of the need of clarifying the physiological mechanisms underlaying learning and memory phenomena as well as of casting light on common degenerative processes, like temporal lobe epilepsy (Margerison and Corsellis, 1966) and Alzheimer's disease (Palmer et al., 1986) that largely affect the hippocampus.

2.2 ANATOMICAL ORGANIZATION

In mammals the hippocampal structure can be considered as the paradigm of a simple cortex, since it consists primarily of one basic cell type and its associated interneurons. Unlike the case of many regions of the cerebral cortex, the cell bodies of the principal hippocampal neurons are densely packed together in one layer, giving rise to two U-shaped structures that constitute the *cornu ammonis* (or hippocampus proper) and the *area dentata* (or dentate gyrus) (see Fig. 3 showing a transverse section of the hippocampus).

Lorente de No (1934) divided the hippocampus proper in 4 fields: CA1-4 ("CA" standing for *cornu ammonis*), which comprises the pyramidal neurons (socalled because of the shape of their cell body) and non-pyramidal neurons, namely the interneurons. Each field is characterized by the specificity of the input/output connections of the pyramidal cells (see Fig. 3).

The most developed fields in the hippocampus proper are CA1 and CA3 (as CA2 and CA4 according to Lorente de No, 1934, can be considered as modifications of CA3 and include a low number of cells). In both fields the cell bodies of pyramidal neurons lay packed in one layer (*stratum pyramidale*, SP). CA1 neurons are smaller and more densely packed in comparison with CA3 ones. They also differ for other functional aspects which will be discussed later. The majority of dendrites develops in one direction, e.g. in CA1 towards the ventral part of the hippocampal formation, while the axons are found to develop in the opposite one. At the base of the axons there are also some dendritic processes. The dendritic tree and axonal processes of the neurons stem out of the cell bodies layer and make contacts with incoming fibers or grow towards further targets at the level of different layers. These layers develop parallel to SP, and in CA1, they can be divided in dorsal and ventral with respect to the SP. The dorsal ones are called *alveus* and *stratum oriens*, and the ventral ones *Schaffer* or *commissural*, *stratum radiatum* (SR) and *lacunosum moleculare* (LM) (see Fig. 4).

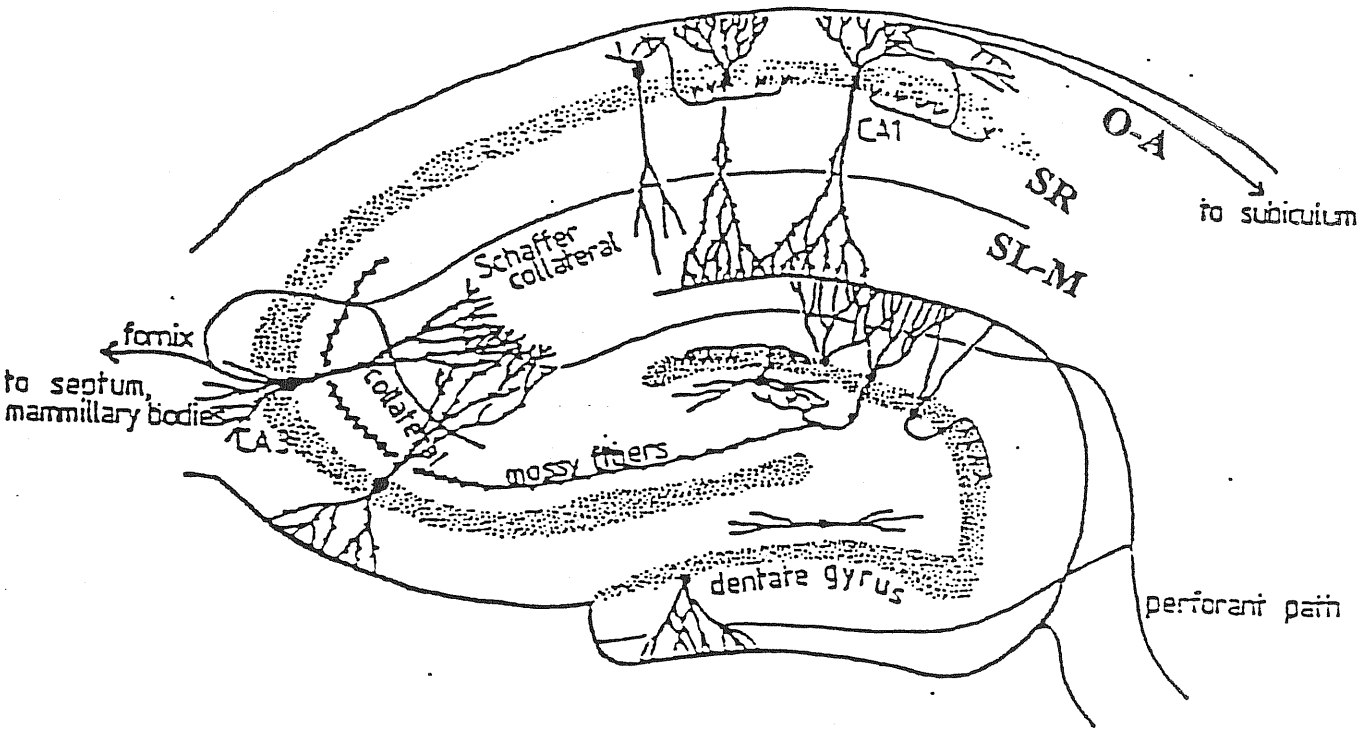


Fig. 3 Simplified drawing of transverse section of the hippocampus with the main *strata* and pathways outlined. **O-A**, stratum oriens-alveus; **SR**, stratum radiatum; **SL-M** stratum lacunosum-moleculare (Rolls and O'Mara, 1993).

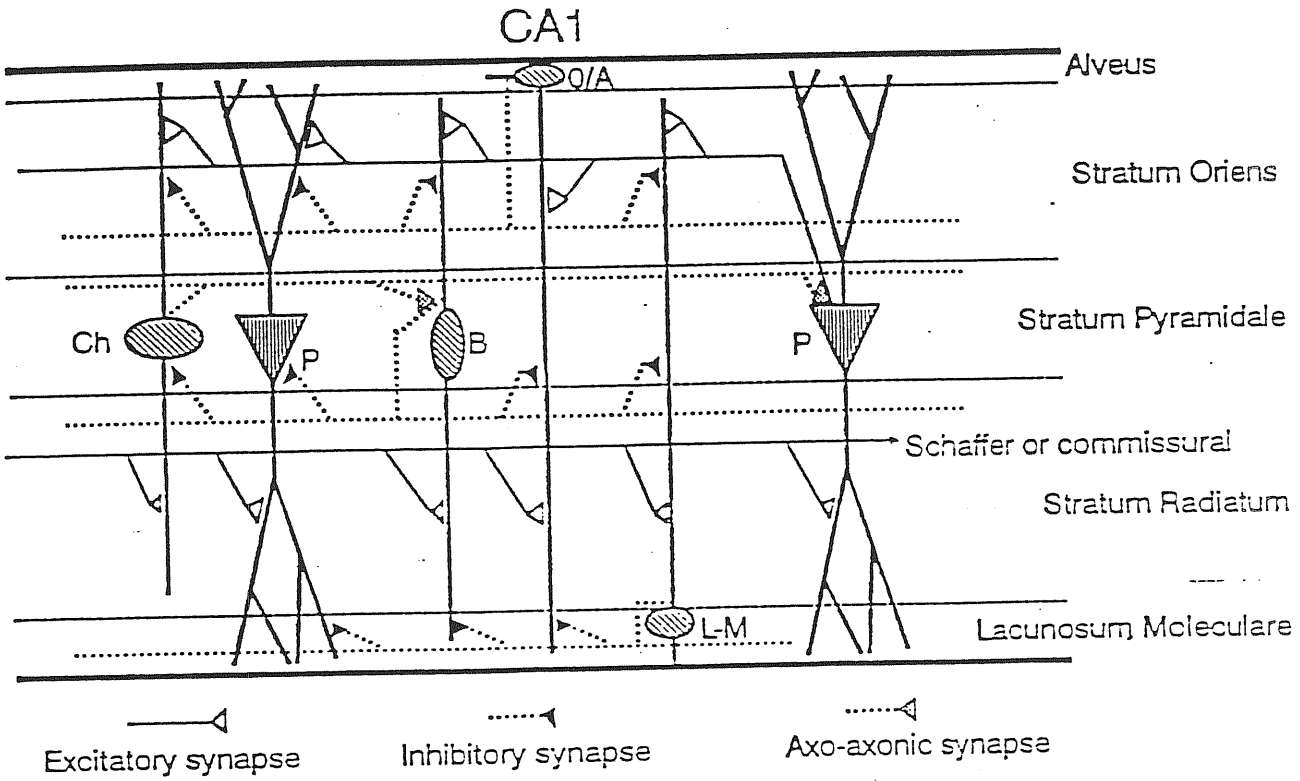


Fig. 4 Simplified schematic representation of the different types of cells and their connections in **CA1** area of the hippocampus. **Ch**, chandelier cell; **P**, pyramidal cell; **O**, oriens interneuron; **R**, radiatum interneuron; **B**, basket cell; **O/A**, oriens/alveus interneuron; **L-M**, lacunosum-moleculare interneuron. Broken lines indicate the laminar boundaries. Thin continuos lines represent excitatory axonal branches. Dotted lines represent inhibitory axonal branches. Empty open triangles represent excitatory; solid triangles represent inhibitory connections; speckled triangles represent axo-axonic synapses (Bernard and Wheal, 1994).

The *area dentata* has three main layers: the *granule* layer containing the densely packed cell bodies of the granule cells; the *molecular* layer formed by the apical dendrites of the granule cells and their afferents; the *polymorph* layer in the hilus of the fascia dentata, which also contains non-granule cells as the basket cells).

2.3 HIPPOCAMPAL CA1 PATHWAYS: EXCITATORY AND INHIBITORY

The principal pathways of the hippocampus are: the septohippocampal, the Schaffer collateral and the perforant pathways. In *stratum oriens*, the fibers of the septohippocampal pathway make cholinergic synapses onto the dendrites of pyramidal neurons branching at the base of the axonal processes. The septal neurons also send a GABAergic input to CA1 GABAergic interneurons which in turn contact pyramidal neurons. This inhibitory effect on inhibition (i.e. disinhibition) leads to excitation of pyramidal neurons.

The major excitatory drive arriving at CA1 dendritic spines is through the Schaffer collaterals (axonal branches from ipsilateral CA3 pyramidal cells) and the commissural fibres (axonal branching from contralateral CA3 pyramidal cells), both present in the same layer. It has been shown (Sayer et al., 1989) that there are also other collaterals developing in the SR, ortogonally to the previous ones (Andersen, 1990), leading to an overall number of about 5 synaptic contacts between a single CA3 pyramidal neuron and a single CA1 neuron (Bernard and Wheal, 1994).

Another excitatory input coming from areas external to the hippocampal formation is the glutamatergic drive from the entorhinal cortex (Jones et al., 1992). These fibers were previously found to connect to the *dentate gyrus* and after a disynaptic circuit to synapse onto CA1 neurons. Only recently entorhinal neurons were also discovered to contact directly CA1 neurons (Witter et al., 1988).

There is also electrophysiological evidence for CA1-CA1 pyramidal neuron connections at the level of the *stratum oriens* (Thomson and Radpour, 1991), but in the slice preparation the calculated probability of transmission for these synapses is quite low (Bernard and Wheal, 1994).

The inhibitory drive to CA1 pyramidal neurons is due to activation of local non-pyramidal cells. The large majority of non-pyramidal neurons is identified on the basis of their glutamate-decarboxylase (GAD) immunoreactivity (Seres and Ribak, 1983), intrisic electrophysiological properties (Schwartzkroin and Kunkel, 1985) and ability to induce inhibitory postsynaptic potentials (IPSPs) in the pyramidal cell they contact (Knowles and Schwartzkroin, 1981). The activity of interneurons is usually considered as inhibitory, even if there is some evidence for the presence of non-inhibitory interneurons (Knowles and Schwartzkroin, 1981; Woodson et al., 1989).

The interneurons in the hippocampus can be divided into at least three families: the basket cells, which synapse on the soma of pyramidal and dentate granule cells; the chandelier cells, which terminate on the initial axon segment of pyramidal neurons in CA1 region (Bernard and Wheal, 1994); and a large number of stellate cells, which are found in the apical and basal dendritic areas of the CA1 and CA3 region.

3. EXCITATORY AND INHIBITORY NEUROTRANSMITTERS

3.1 GLUTAMATE

Glutamate is the major excitatory neurotransmitter in the CNS of vertebrates. The type of response that glutamate can elicit depends on the receptors to which it binds. There are two main families of glutamate receptor: the ionotropic one, which contains a channel as an integral part, and the metabotropic one, which is a receptor coupled via a G-protein system to a separate channel. The ionotropic receptors can be subdivided in two main classes according to their

molecular structure and their different selectivity for agonists (Seeburg, 1993): the N-methyl-D-aspartate (NMDA) and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (non-NMDA or AMPA).

The NMDA receptors are slowly activated and are mainly permeable to cations such as Na^+ and Ca^{2+} (Asher and Nowak, 1988) through its pore. Even if the NMDA receptor is a ligand gated channel, it is also sensitive to voltage because of the voltage dependent block exerted by Mg^{2+}. Moreover, the activation of the channel requires glycine as co agonist (Johnson and Asher, 1987). At resting membrane potential, after opening of the channel by NMDA, Mg^{2+} binds inside the pore and blocks it. Only when the membrane is depolarized, for example by the influx of cations through other receptors, the Mg^{2+} block is relieved and Ca^{2+} and Na^+ ions can enter into the cell.The fact that the block of Mg^{2+} can be relieved by postsynaptic depolarization makes this receptor to act as an "integrator" of pre and postsynaptic activity, therefore its modulation become very important during phenomena of synaptic plasticity.

The AMPA receptors are activated with fast kinetics (in the order of ms) and are mainly conducting Na^+ and K^+ since they are usually characterized by very low Ca^{2+} permeability (Iino et al., 1990). Their conductance is lower (1-15 pS) in comparison with the NMDA receptor one (40-50 pS). NMDA and AMPA mediated responses are selectively antagonized by the presence of 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and 6-cyano-7-notroquinoxaline 2,3-dione (CNQX), respectively.

The glutamate mediated postsynaptic potentials can be usually divided in two main phases: an early fast rising phase during which the AMPA component prevails and a later slow falling phase mainly mediated by NMDA receptors. There can be several ways to explain two so different kinetics of NMDA and non-NMDA mediated components, since the physiological agonist is the same for both. One possible hypothesis (Jonas and Spruston, 1994) is the following: the rise of

glutamate concentration in the synaptic cleft has to be very rapid and also its diffusion so activation and deactivation of AMPA receptors are both very rapid. Moreover, the duration of AMPA receptor mediated responses is strongly limited by its desensitization (Trussel and Fischbach, 1989). These characteristics make the late component of glutamatergic responses to be mainly NMDA mediated permitting a strong modulation depending on the voltage history of the membrane and Mg^{2+} block. Since slowly developing responses have a higher probability to add one to another, modulation of this component may lead to a strong integration of several synaptic inputs.

3.2 MODULATION OF GLUTAMATE MEDIATED RESPONSES

Since the AMPA receptor is so strongly desensitizing, drugs such as cyclothiazide which block desensitization upregulate glutamate mediated activity (Vyklicky et al., 1991). The NMDA receptor has several modulatory sites, as Mg^{2+} is blocking the channel from the inner part of the channel (Scatton, 1993), Zn^{2+} has been found to control receptor function through its binding to a specific site at the mouth of the pore (Scatton, 1993). NMDA receptor mediated responses are also inhibited by high concentrations of H^+ which affect the probability of opening of the channel (Scatton, 1993). The NMDA receptor can be positively regulated by glycine and polyamines, which act via distinct binding sites. Activation of PKC leads to modification of the binding site for Mg^{2+}: in this way it decreases the ability by Mg^{2+} to block the channel (Chen and Huang, 1992).

3.3 GABA

Synaptic inhibition in the mammmalian brain is mainly due to the release of γ-amino-butyric-acid (GABA) which binds to specific receptors termed: $GABA_A$ and $GABA_B$.

$GABA_A$ receptors which mediate fast inhibitory postsynaptic potentials (IPSPs) are made up by five subunits (Macdonald and Olsen, 1994) that form a channel permeable to Cl^-. On the other hand, $GABA_B$ receptors, which mediate slow inhibitory activity, belong to the family of seven-

transmembrane spanning metabotropic receptors coupled with a channel via a G-protein. Another feature of $GABA_B$ receptors is their localization: they are present both pre and postsynaptically. In the hippocampus the inhibitory action of postsynaptic $GABA_B$ receptor mediated responses is obtained with activation of a K^+ conductance (Gähwiler and Brown, 1985) causing hyperpolarization of the membrane potential of the cell. In other regions of the CNS a change in presynaptic Ca^{2+} conductance has been described, but in the hippocampus any change in Ca^{2+} influx is considered to be secondary to the K^+ conductance activation (Gähwiler and Brown, 1985). The activation of $GABA_B$ receptors at the presynaptic terminal leads to a decrease in transmitter release. If the terminal is GABAergic, $GABA_B$ receptors activation can lead to two different effects: either the increase and/or the decrease of inhibition, depending on whether those receptors are located at the pre or postsynaptic side. This fact makes $GABA_B$ receptors to act as autoreceptors limiting their own activation when a massive release of GABA is occurring.

It has been observed that in order to elicit a postsynaptic $GABA_B$ mediated response, stimuli of higher intensity are required when compared with the ones eliciting $GABA_A$ mediated responses. $GABA_B$ receptors are perhaps clustered in a postsynaptic area not as easily accessible to GABA as the $GABA_A$ receptor area (Mody et al., 1994). In this case only when a large amount of GABA is released, $GABA_B$ receptors could be activated.

There is also evidence for a diverse cellular localization of the two classes of receptors. $GABA_A$ receptors are abundant on neuronal somata (Newberry and Nicoll, 1985) whereas $GABA_B$ receptors are preferentially located on pyramidal cell dendrites (Newberry and Nicoll, 1985). These features could account for the slower onset of $GABA_B$ mediated responses, due to filtering by the dendritic tree (Spruston et al., 1994).

3.4 MODULATION OF GABA-MEDIATED RESPONSES

Since desensitization appears to play little role in shaping $GABA_A$ mediated spontaneous currents (Borst et al., 1994) and GABA-uptake inhibitors do not alter their current kinetics (Otis and Mody, 1992), the modulation of GABAergic drive can be mainly accomplished by either a presynaptic change in the amount of neurotransmitter released or a postsynaptic change in the receptor kinetic properties. Several drugs can affect GABAergic drive such as opiates, which act at the presynaptic site (Thomson et al., 1993) while barbiturates, benzodiazepines and volatile anaesthetics (Otis and Mody, 1992; Mody et al., 1991) bind to the GABA receptor modifying directly its kinetic properties. Furthermore, GABA channel activation may be modified by agents that increase phosphorylation. The activation of PKC reduces responses mediated by both $GABA_A$ and $GABA_B$ receptors (Leidenheimer et al., 1992; Andrade et al., 1986).

4. LONG TERM POTENTIATION

4.1 DEFINITION

Long term potentiation (LTP) is defined as a stable, relatively long lasting (from 1 hour up to months) increase in the magnitude of a post-synaptic response to a stimulus of constant amplitude. It was initially observed in the dentate gyrus of the hippocampus (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973) and it has now been described in other hippocampal regions as well as in a variety of other brain structures: from the entorhinal to the somatosensory cortex (Racine et al, 1983; Gerren and Weinberger, 1983; Komatsu et al., 1981; Voronin, 1985) and even in the cerebellum (Ito, 1983).

LTP is a phenomenon present in phylogenetically different species, including lower vertebrates (Lewis and Teyler, 1986a) and invertebrates (Hawkins et al., 1993), even though the experimental protocols to initiate LTP may be different and may operate via mechanism different from those of the mammals. The simplest form of potentiation is called **facilitation**: this is a presynaptic

phenomenon due to the change in Ca^{2+}concentration which happens at the presynaptic terminal following repetitive activation and the increase in Ca^{2+} concentration caused by one stimulus can summate with the one elicited by the following, leading to a larger amount of neurotransmitter released.

4.2 LTP IN THE HIPPOCAMPUS

In order to induce a synaptic potentiation in the hippocampus, a particular protocol has to be followed. A tetanic stimulation, namely one or more trains of extracellular stimuli at high frequency (100 Hz), has to be delivered to the pathway interested. This pattern of stimulation has been shown *in vitro* to mimic the enhancement of electrical activity recorded *in vivo* in the hippocampus of animals during behavioural training (Laroche and Bloch, 1982).

The tetanic stimulation may induce the expression of a repertoir of distinct forms of facilitation according to their duration: post-tetanic potentiation (PTP), short term potentiation (STP) and finally LTP.

PTP is an immediate change in synaptic efficacy which can be observed just after the tetanic stimulation, it lasts more than pure presynaptic facilitation and it typically decays over the first 20 min from the stimulation (Bliss and Collingridge, 1993).

STP may follow and is considered to be over within 40 minutes (Malenka and Nicoll, 1993).

LTP starts after 40 minutes from the tetanic stimulation and it can last several hours *in vitro* or even days and weeks *in vivo* (Bliss and Lomo, 1973; Bliss and Collingridge, 1993).

4.3 MECHANISMS

A lasting change in the synaptic responses to a constant stimulus may result from the modulation either at the presynaptic site (namely a change in the quantity of neurotransmitter released) and/or

at the postsynaptic site, a change in the effect that a constant amount of transmitter elicits after binding to its receptor). Moreover, some retrograde messenger may be sent from the postsynaptic side to the presynaptic one in order to participate in synaptic plasticity.

The synaptic potentiation can be divided into two principal categories depending on whether the NMDA receptor are or are not necessary to be activated during the induction. The first case concerns LTP in the Schaffer collateral-CA1 pyramidal neurons and perforant pathway-granule cells synapse. The latter concerns the Mossy fiber-CA3 pyramidal neurons. Here, CA1 LTP will be considered.

4.3.1 Induction

There are still controversial lines of evidence about the synaptic mechanism which plays the major role during the induction of LTP. There is evidence that, in order to induce LTP in physiological conditions, the following postsynaptic requirements are necessary but not sufficient to be fullfilled:

1. NMDA receptors activation
2. increase in intracellular Ca^{2+} concentration
3. phosphokynase C (PKC) activation

High frequency stimulation leads to a strong increase in glutamate release, which, due to the massive activation of AMPA receptors, depolarizes the membrane of the neuron. When the membrane is depolarized, Mg^{2+} dependent block can be removed and Ca^{2+} allowed to enter into the cell. The intracellular increase in free Ca^{2+} will then activate a cascade of second messengers, among which PKC, leading to other substrate phosphorilation.

4.3.2 Expression

The mechanisms involved in the expression of LTP are still controversial. Evidence both for pre and postsynaptic involvement has been obtained. The presynaptic mechanisms that can increase the amount of transmitter released might include any element involved in Ca^{2+} homeostasis. Modulation of Ca^{2+} entering affecting Ca^{2+} or K^+ channels as well as any other metabolic element involved in the pathway triggered by Ca^{2+} entry and leading to the docking and release of the vescicles in the synaptic cleft. Quantal analysis studies has shown an increase in the mean number of quanta, i.e. number of vescicles, released after LTP induction (Malinow and Tsien, 1990; Malinow, 1991; Malgaroli and Tsien, 1992).

It is likely that the postsynaptic component of LTP involves alterations in the properties and/or numbers of the ion channels activated during the process. An enhancement of the postsynaptic component of both AMPA and NMDA mediated responses has been described. There are also some preliminary data which suggest the involvement of a phosphorylation of AMPA and NMDA receptors, by PKA and PKC respectively, leading to an enhancement in their mediated responses.

5. AIM OF THE PROJECT

The aim of the project was to investigate whether the neuropeptide TRH had any significant effect on CA1 pyramidal neurons in the hippocampus and more precisely to examine whether these effects were affecting:

1. **intrinsic membrane properties** of CA1 pyramidal neurons, modifying eventual conductances active either at rest or at more depolarized potentials, with particular interest in K^+, Ca^{2+} and K^+-Ca^{2+} dependent conductances.

2. **neurotransmitters activity** since neuropeptides are known to exert modulatory action on various neurotransmitters. We wanted to study the effect of TRH effect on excitatory and inhibitory neurotransmitters present in the hippocampus, e.g. glutamate and GABA, using endogenous and exogenous agonists. We wanted to further investigate the main site of action of TRH, namely at postsynaptic (pyramidal side) or presynaptic (fiber-interneuronal) circuit.

3. **short and long term modulation**: if TRH can modulate responses to neurotransmitter, it was of interest to investigate if it could also affect short and long term modulation of synaptic activity induced by high frequency stimulation.

METHODS

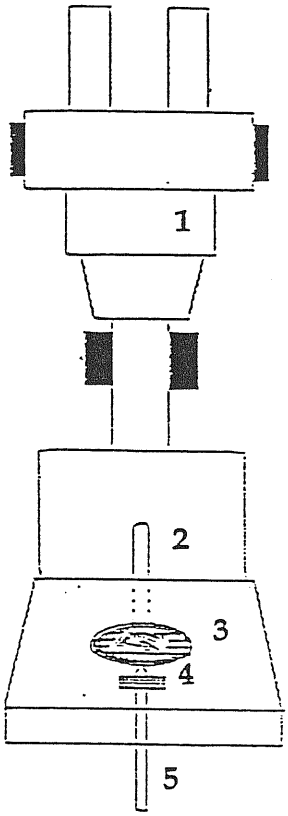
1. SLICE PREPARATION

Adult (16-38 days) Wistar rats were housed at constant temperature (20°C) and humidity with unrestricted access to standard chow and water. Animals anaesthetized using diethylether (Merck) were decapitated. Their brain was rapidly removed from the skull and submerged into artificial cerebrospinal fluid (ACSF) of the following composition (mM):

NaCl 126, KCl 3.5, $CaCl_2$ 2, NaH_2PO_4 1.2, $MgCl_2*6H_2O$ 1.3, $NaHCO_3$ 25, glucose 11.

The ACSF was equilibrated with 95% O_2 and 5% CO_2 to reach pH of 7.3-7.4 at room temperature (about 22° C). The two hemispheres of the brain were separated with a sharp blade and the two hippocampi carefully isolated. Each one of them was laid on an Agar bed (4 gr Agar in 100 ml of a physiological saline obtained diluting 9 g of NaCl in 1000 ml distilled water) and transversally cut into slices with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co.Ltd.). Slices (400-500 µm thick) were allowed to recover in oxygenated ACSF at room temperature for about 1 hour before use. A single slice was transferred to a recording chamber (about 1 ml volume) which contained a plastic ring with a nylon mesh on which the slice was laid (Fig. 5 A) and continuously superfused at room temperature with ACSF (saturated with oxygen) at a rate of 2.5-4 ml per minute. The fluid was either gravity fed or electrically pumped. The bath was transilluminated by optic fibers and the layer of CA1 pyramidal cells unambiguously identified (see Fig. 5 B). All drugs were delivered by bath application.

A



B

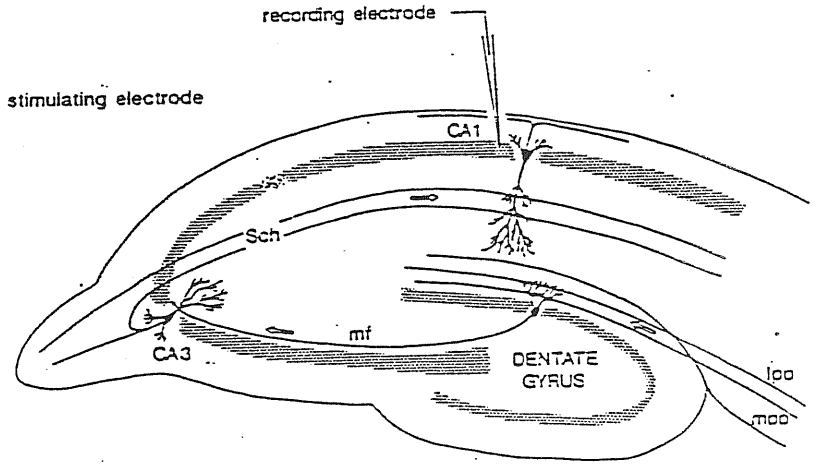


Fig. 5 A: diagram of the recording chamber arrangement: **1** microscope, **2** inlet tubing, **3** nylon mesh with hippocampus slice, **4** paper wick, **5** outlet tubing.
B: Schematic diagram of the rat hippocampal slice preparation: **CA1** and **CA3** regions, lateral (**lpp**) and medial perforant paths (**mpp**), mossy fibers (**mf**) and Schaffer collateral-commissural (**Sch**) pathway (Collingridge and Davies, 1989) .

2. 1 INTRACELLULAR TECHNIQUES

Intracellular recordings, current injection, discontinuous current clamp (DCC) and single electrode voltage clamp (SEVC) were conducted using an Axoclamp 2A amplifier (Axon Inst. Burlingame, CA). A microelectrode with a resistance of 50-110 MΩ was pulled by a Brown-Flaming Micropipette Puller (Sutter Inst. San Francisco, CA) using borosilicate glass tubing, 1 and 1.5 mm O.D., (Clark Electromedical Instruments) and filled with 3 M KCl, 3 M CsCl, 4 M K-acetate (pH adjusted to 7.2 with glacial acetic acid) or 2M K-methylsulphate. It was then inserted into a holder filled with 3 M KCl connected through a pin to the headstage of the AxoClamp.

BRIDGE MODE

Cells were impaled in bridge mode (socalled because it refers to the original Wheatstone bridge circuit once used to balance the voltage drop of the electrode and now replaced by an operational amplifier) after having properly compensated the capacitance of the pipette, balanced the bridge and offset the voltage. The bathing solution of the recording chamber was grounded via a paper wick using a silver-silver chloride pellet. Searching for cells was done after positioning the electrode in the CA1 region and applying short (10-15 ms) hyperpolarizing pulses of 0.3-0.4 nA amplitude at high frequency (10 Hz) while advancing through the slice in small steps with the aid of a Narashige micromanipulator. When the surface of a cell was reached, a sudden increase in resistance was detected on the oscilloscope and the impalement was obtained using the "clear" switch of the AxoClamp through which a large, transient current can be sent down the pipette making its tip to oscillate and enter the cell. Just after impalement a negative steady current (about -0.3 nA) was injected into the cell, to stabilize recording.

During all the current clamp experiments two pulses of current, one second apart, an hyperpolarizing and a depolarizing one (about 0.1 and 0.3 nA amplitude, 400 ms and 300 ms

duration respectively) at a frequency of 0.1 Hz were routinely applied to the cell for continuous monitoring of the input resistance (Rm).

DCC

The DCC mode was switched on after optimizing capacitance neutralization as well as sampling rate in order to obtain a full decay of voltage transients monitored separately on a second oscilloscope. This technique allowed more reliable measurement of the membrane potential (Vm) even in the presence of small changes in microelectrode resistance since sampling (3-4 KHz) of the membrane voltage is performed after transient modifications of potential, due to electrode resistance, have dissipated. If requested by the protocol, the DCC mode was then followed by discontinuous SEVC.

dSEVC

This procedure was started by ensuring that the clamp level corresponded to resting membrane potential. The gain control and the anti-alias filter were set to a minimum and a -10 mV step was applied repetitively. At this point the gain was gradually increased to ≥1 nA/mV with fine tuning of the phase shift for current/voltage switching until a satisfactory voltage control of the onset/offset of the step was achieved coincident with a smooth current response. In the recorded currents leak subtraction was performed on the basis of the I/V curve obtained at potentials near rest at which voltage activated conductances were supposed to be absent.

Only cells in good condition were studied, with initial Vm of -70 mV and spike amplitude exceeding 85 mV.

2. 2 EXTRACELLULAR STIMULATION

Extracellular stimulation of fibers or cells in different strata was performed with twisted tungsten wires (insulated except at their tip) placed approximately 100-200 µm away from the pyramidal neurons from which recordings were obtained. The choice of the stratum to stimulate depended

on the circuit studied: the *stratum radiatum* (SR), in the case of the Schaffer collateral fibers and the *stratum lacunosum-moleculare* (SLM) or *pyramidale* (SP) in the case of interneurons. Electrical pulses of 50-80 μs duration, 20-70 V amplitude were delivered at 0.05 or 0.1 Hz frequency in order to elicit glutamatergic or GABAergic responses. In the case of glutamatergic responses such a frequency (0.05 Hz) was chosen to rule out modulatory effects on neurotransmitter release depending on the rate of stimulation (Perouansky and Yaari, 1993). The amplitude of the stimulus to elicit postsynaptic potentials was submaximal in order to observe any upmodulation. In the case of NMDA receptor mediated potentials the stimulus was adjusted to elicit minimal responses, namely responses due to the activation of a very limited number of presynaptic fibers.

In the case of the induction of STP and LTP the Schaffer collaterals fibers were stimulated with 4 trains of 100 Hz and 1 sec duration. Each train was delivered a time interval of 30 sec. The extracellular stimulations preceding and following the trains were delivered at 0.05 Hz.

3. DATA ANALYSIS

Current and voltage responses were recorded both on a GOULD chart recorder and on a video cassette recorder (PHILIPS) using a VR-10A CRC digital data recorder (Instrutech-Corporation). In the case of the evoked potentials data were either online or offline acquired on a PC using PCLAMP 6.1 software.

The decay time of EPSPs was calculated with a monoexponential fit (between 90 and 10% of the peak amplitude) forced to the baseline. The EPSP area was calculated as the time integral between the start and the end of the response. Whenever spike threshold was reached, the EPSP amplitude was measured at the foot of the first spike and the corresponding area was calculated after subtraction of the component underlying the spike(s). Control EPSP/IPSPs were measured by averaging 10-20 responses preceding TRH application. Since the effect of TRH on

glutamatergic potentials was usually manifested only during the first 2-3 min after its application, data for TRH treatment were obtained by ignoring the first response after applying the peptide and averaging the subsequent four responses. In the case of GABAergic IPSPs, the traces shown were obtained after averaging of 10-20 traces in control and 10-20 responses in TRH. In the case of STP/LTP experiments the single evoked potentials were averaged in batches of three and measures were performed on the latter. Measurements of peak amplitudes, areas and rise time (10-90%) were performed with the PCLAMP 6.1 program. During this protocol, since only picrotoxin was delivered to reduce part of the GABAergic drive, the EPSPs was much faster than those pharmacologically isolated as previously mentioned. In the case in which the EPSPs were reaching threshold for spiking, it was often difficult to measure their areas. In this instance the area was approximated to the maximal value measured in control, before the induction of STP/LTP. Note that this method was leading to an underestimation of the phenomenon.

Results are given as mean ± s.e.m.; statistical analysis was performed using Student *t* test in the case of agonist bath application and with the signed ranks Wilcoxon test in the case of evoked potentials.

RESULTS

1. EFFECT OF TRH ON INTRINSIC CONDUCTANCES

1.1 RESTING CONDUCTANCES

Bath application of TRH (5-20μM) did not significantly change Vm of any neurons tested (n=76). On a sample of 32 cells with Vm of -61±1 mV TRH elicited a depolarization of 1±0.44 mV. Resting input resistance (continuously monitored as described in the methods) was not changed by TRH, since it was 69±6MΩ in contol *vs* 68±6MΩ.

1.2 AFTER HYPERPOLARIZING POTENTIAL (AHP)

In hippocampal pyramidal cells action potentials are usually followed by an after hyperpolarization of which at least two components can be distinguished (Storm,1990): a fast one, lasting few milliseconds, and a slow one which lasts for more than one second. The first can follow each spike and takes part in the repolarization phase of the action potential, whilst the latter is more easily detectable after a burst of spikes. This after-hyperpolarization is principally due to various Ca^{2+}-dependent K^+ conductances activated during spiking.

Data were obtained, in DCC mode, using K-acetate (4 M) filled electrodes (70-110 MΩ) in neurons (n=4) with a membrane potential of -74±1.55 mV and receiving hyperpolarizing (amplitude of about 0.1 nA, duration of 400 ms) and depolarizing steps (0.1-0.2 nA, 250 ms). The stimuli were delivered at 0.071 Hz with an interpulse delay of about 2 seconds.

Application of TRH (5μM) reduced the AHP in all the neurons, even if the V_m before the spike train and the number of spikes induced by the depolarizing step were kept constant (Fig. 6 A and B). Of the two components the slow one, measured at 500 ms after the end of the pulse, was

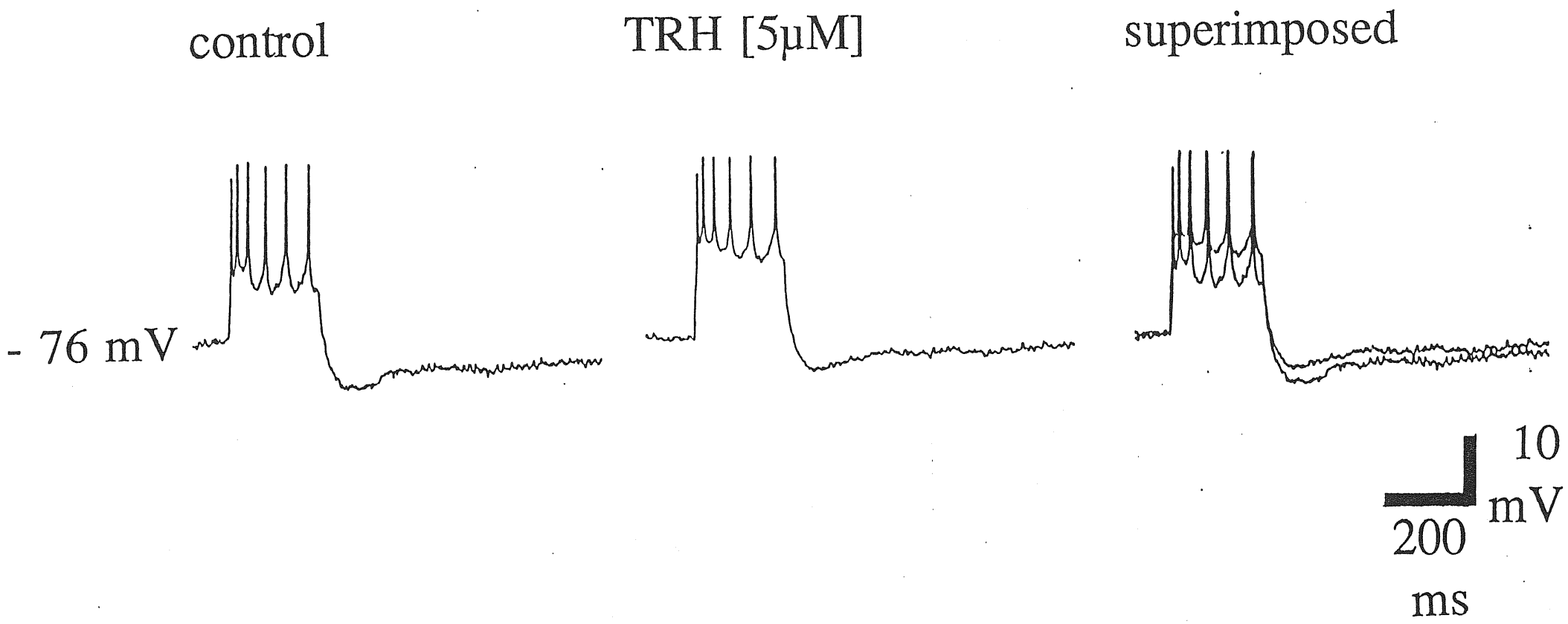


Fig. 6 A Effect of TRH on after-hyperpolarization. Voltage traces showing the membrane potential responses to depolarizing steps of current (0.18 nA) of the duration of 250 ms. Depolarizations elicited a train of spikes followed by a long after-hyperpolarization. 5 µM TRH was decreasing the amplitude of that hyperpolarization (see superimposed traces).Stimuli delivered at 0.071Hz with interpulse delay of 2s.

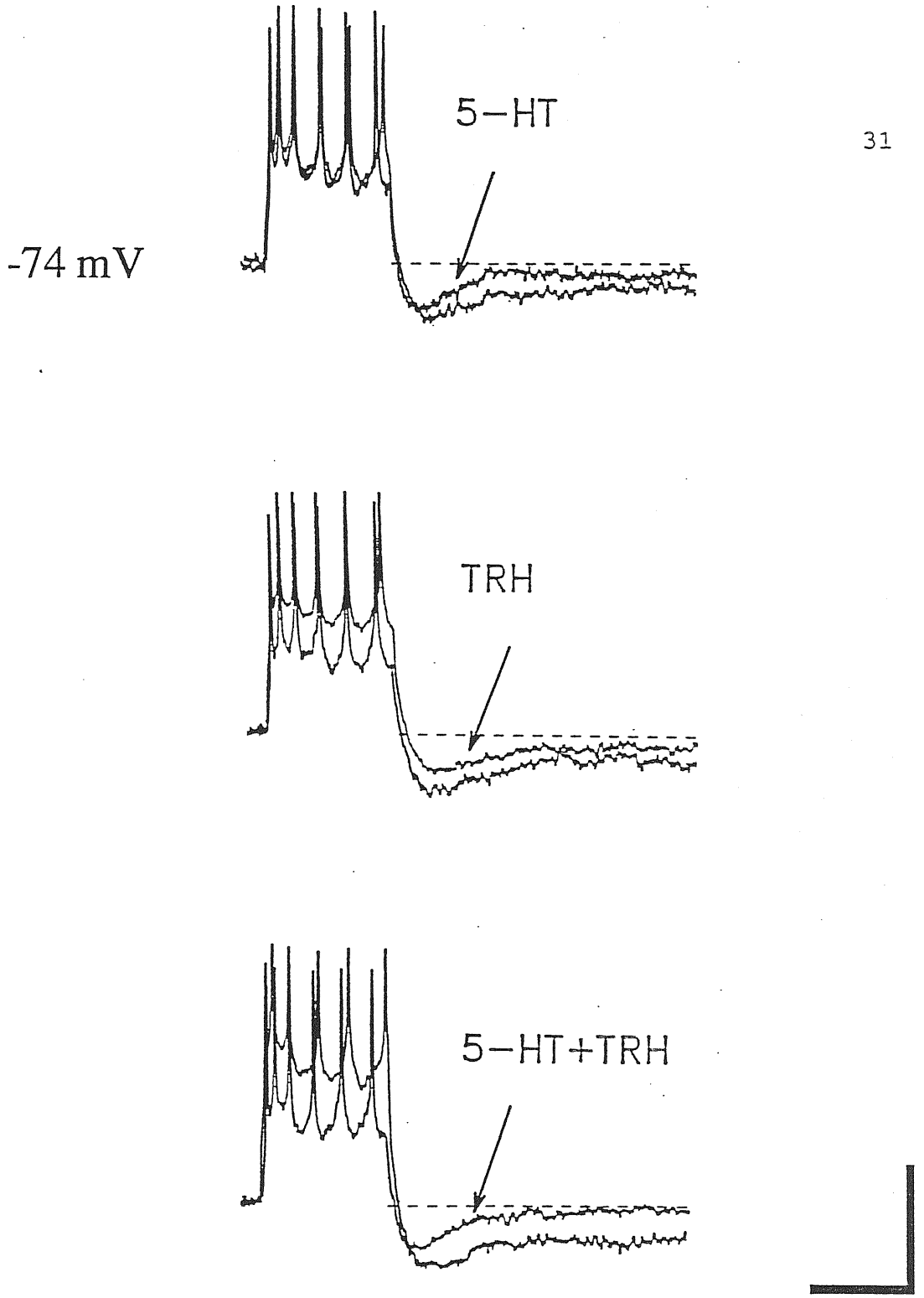


Fig. 6 B Effect of 5HT and TRH (alone and in combination) on the AHP of hippocampal neurons. Control and drug application (arrow) tracings are superimposed. Top row: control condition and after 5 min in the presence of 5μM 5HT. Middle row: after 15 min washout from 5HT and after 10 min in the presence of 5 μM TRH. Bottom row: after 20 min washout from TRH and co-application of 5μM TRH (10 min). Note that following co-application of the two drugs, the late AHP is blocked. Calibrations: 15 mV, 250 ms

more affected with a fractional amplitude of 0.67±0.14 when compared to the control one in ACSF. The peak fractional amplitude of the fast component was also diminished (0.77±0.02). The interspike interval was not changed; note also that, although TRH did not alter input resistance at rest as previously mentioned, this peptide increased the amplitude of the depolarizing envelope elicited by positive current pulses (see Fig. 6 A and B). This observation suggests that a yet unidentified voltage sensitive conductance operating at relatively depolarized membrane potential might have been modulated by TRH. Further attempt was done to identify the nature of this conductance. On the same four cells the effect of both TRH and 5-hydroxytryptamine (5HT) (Fig. 6 B), which is known to depress the AHP by blocking a Ca^{2+} dependent K^+ conductance (Andrade and Chaput, 1991), together with TRH was decreasing the AHP. 5HT (5μM) alone decreased the AHP amplitude to 0.77±0.011 fractional response of control at the peak and to o0.30±0.10 at 500 ms after the end of the depolarizing pulse. When 5HT and TRH were coapplied the peak fractional amplitude was reduced to 0.58±0.07 and the later phase of the AHP to 0.15±0.1. Note that half maximal doses of the drugs were used and that during coapplication there was an additive effect.

1.3 HIGH VOLTAGE ACTIVATED CALCIUM CURRENTS (HVA-I_{Ca2+})

In order to investigate whether the effect of TRH on the AHP was caused by an indirect modulation of other conductances, Ca^{2+}currents were studied in SEVC mode. To eliminate voltage-activated Na^+currents and the majority of K^+conductances, cells were superfused with a solution containing: 1 μM tetrodotoxin (TTX) , 20 mM tetraethylammonium (TEA), 3 mM Cs^+, 3 mM 4-amino-pyridine (4AP) and the pipette was filled with 3 M CsCl. From a holding potential of -40 mV, hyperpolarizing and depolarizing steps of 10 mV increments in amplitude and 1 second duration were delivered at a frequency of 0.05 Hz. In 6 cells inward currents were measured at steady state (at the end of the pulse) and started to develop at -30 mV reaching a

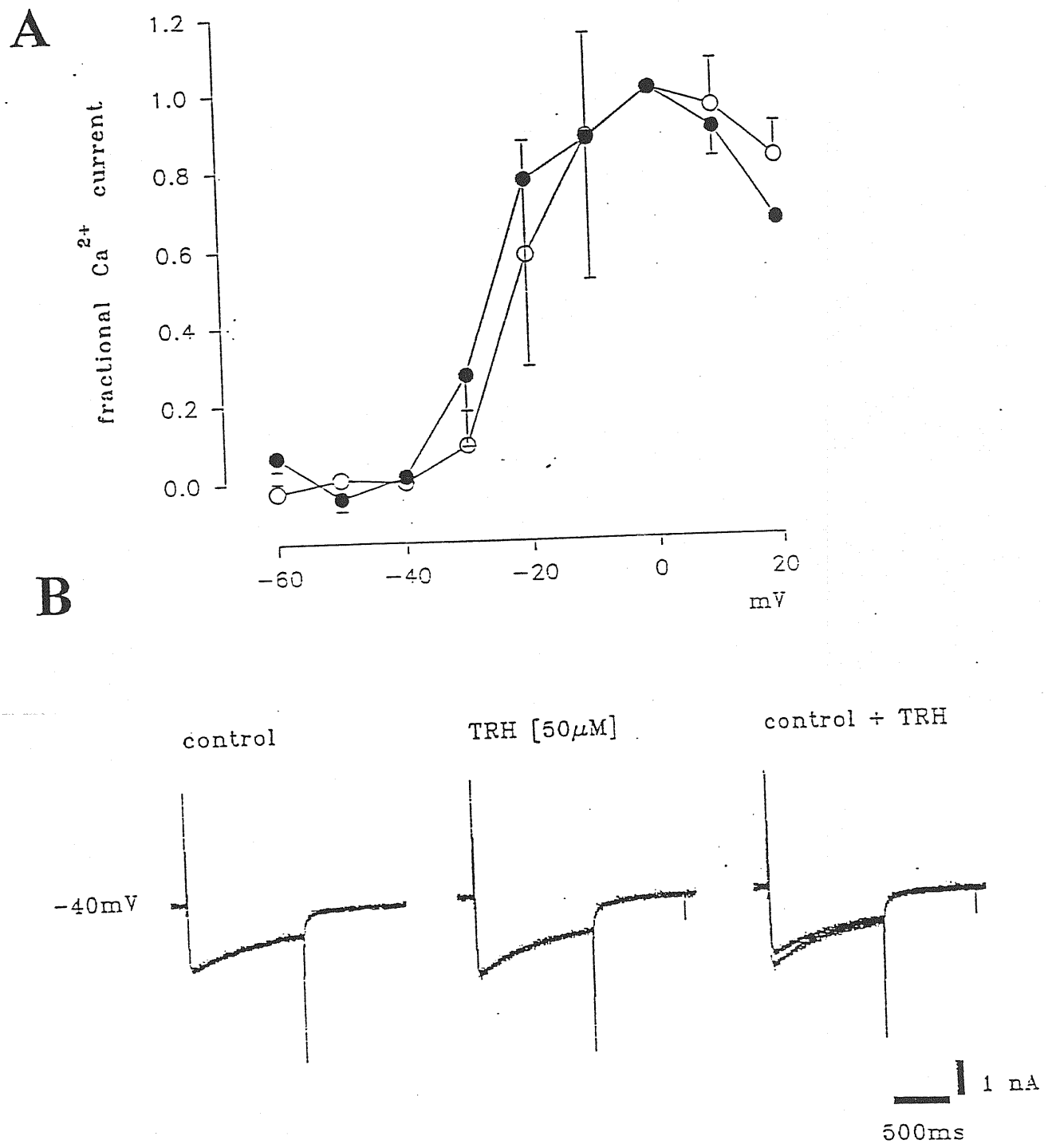


Fig. 7 Effect of TRH on Ca^{2+}currents. Voltage clamp Ca^{2+}currents recorded from a neuron at a holding potential of -40 mV in the presence of 1 μM TTX, 20 mM TEA, 3 mM Cs^{+}, 3mM 4AP in the solution and 3 M CsCl in the pipette. Steps of 10 mV increments and 1 s duration were applied at 0.05 Hz. A: I/V curve obtained after leak subtraction: a comparison between values measured in control solution (open circles) and in TRH solution (filled circles). Data were normalized *vs* the current value obtained when the membrane was clamped to 0 mV. B: traces of inward current elicited clamping the cell to -10 mV (leak not subtracted).

maximum around 0 mV, as it can be seen from the I/V curve of Fig. 7 A obtained after leak subtraction. TRH (50μM) application did not significantly affect slow Ca^{2+}currents as it can be seen in the plot of Fig. 7 A (open and close circles are control and TRH solution respectivelt) and in the traces of Fig. 7 B (latter were not leak subtracted).

2. MODULATION BY TRH OF GLUTAMATERGIC ACTIVITY

2.1 EVOKED POTENTIALS

Data were obtained from 15 pyramidal neurones recorded at their resting membrane potential (-67±2 mV) throughout each experiment. In order to isolate NMDA receptor-mediated responses, experiments were routinely performed in the presence of 6-cyano-7-notroquinoxaline 2,3-dione (CNQX, 10 μM), bicuculline (20 μM) and CGP 35348 (1 mM), to block synaptic potentials mediated by AMPA/kainate, $GABA_A$ or $GABA_B$ receptors, respectively. The stimulation intensity of the Schaffer collaterals was adjusted to elicit minimal EPSPs, *i.e.* presumably those due to the activation of one or very few afferent fibres (Voronin, 1993) as suggested by their small amplitude (usually about 2 mV) and frequent transmission failure detected in a number of cells.

NMDA-receptor mediated EPSPs.

In control conditions the EPSP amplitude appeared to fluctuate within a relatively narrow range (for instance, 1.3-3.3 mV for the cell of Fig.8 and 0.7-1.8 mV for the cell of Fig. 9), and was fully blocked by 10 μM 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP). Averaging at least 10 control EPSPs from 15 cells produced 1.6±0.1 mV peak amplitude, 353.4±51.3 mV*ms area, and 131.1±13.7 ms decay time constant (τ_d). Note that these small amplitude responses described soforth were recorded in the presence of Mg^{2+} at physiological concentration (1.3 mM).

Fig. 8 shows an example of the effect of TRH (10 μM) on NMDA receptor-mediated EPSPs The control response (see Fig. 8 A) had an average 2.3 mV amplitude and 703 mV*ms area and

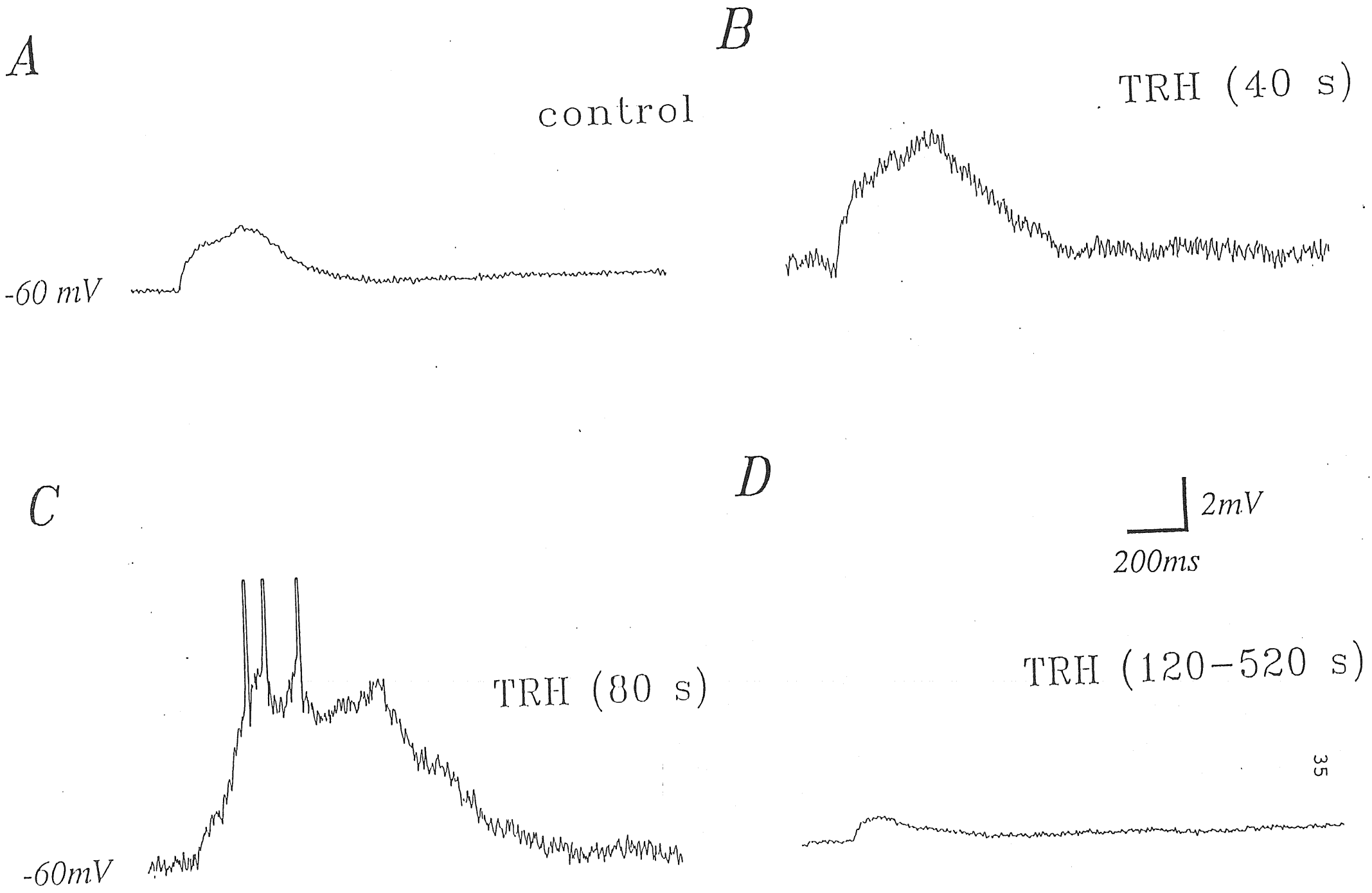

A
control
-60 mV
B
TRH (40 s)
C
TRH (80 s)
-60mV
D
2mV
200ms
TRH (120–520 s)

Fig. 8 Effect of TRH (10μM) on NMDA receptor-mediated EPSPs of CA1 pyramidal neurone.
A: average trace of 18 responses recorded in control solution: the biphasic nature of the EPSP suggests it was generated by activation of two fibres; **B**: single trace recorded after 40 s exposure to THR: note increase in both amplitude and duration; **C**: single trace recorded after 80 s exposure to TRH: cell now reaches firing threshold and generates three spikes (truncated); **D**: average trace of 19 responses recorded over 120-520 s exposure to TRH: note loss of enhancing action and monophasic appearance of EPSP.

probably comprised two summated EPSPs as suggested by its biphasic appearance. Fourty s after TRH application the amplitude grew to 4.7 mV and the area to 1894 mV*ms (199 and 269% of the respective controls; *cf.* Fig. 8 B in which the tracing of a single response is clearly noisier than the averaged control). The largest enhancement (Fig. 8 C) was observed 80 s from the start of TRH application when the EPSP reached firing threshold (spikes truncated). Following continuous application of TRH the EPSP ehancement was not maintained (see Fig. 8 D) and a monophasic EPSP was revealed over the sampled time (120-520 s). After 1 h washout of TRH the EPSP had returned to control value and a further application of TRH reproduced a similar, temporary increase in the NMDA receptor-mediated EPSP. On 15 cells the transient (within 2 min) potentiation by TRH of the EPSP peak amplitude was 34.8±14.5% (p=0.02), while the EPSP area was also significantly augmented (60.8±25.7%; p=0.02). No significant change in τ_d was observed. After 6-7 min continuous application of TRH, peak amplitude, area and τ_d of the NMDA receptor-mediated EPSP were not dissimilar from control (90.0±9.6, 99.6±12.7 and 106.4±8.7% of their respective control). A more detailed representation of the timecourse of TRH action is depicted in the graph of Fig. 9 B, in which the EPSP area was first increased and then returned to baseline level (with a number of failures). The enhancing action of TRH was not accompanied by a detectable change in resting membrane potential or any significant alteration in input resistance (107±5%; n=8). An example of this phenomenon is given by the superimposed tracings of responses before and after addition of TRH (Fig. 9 A): the large potentiation of the e.p.s.p. which reached firing threshold was associated with no alteration in the resting potential level or in the amplitude of the electrotonic potential.

AMPA receptor-mediated EPSPs

In 6 cells it was possible to washout TRH for at least 1 h by bathing cells in control ACSF and then to apply (for approximately 20 min) a solution (containing bicuculline and CGP 35348) in

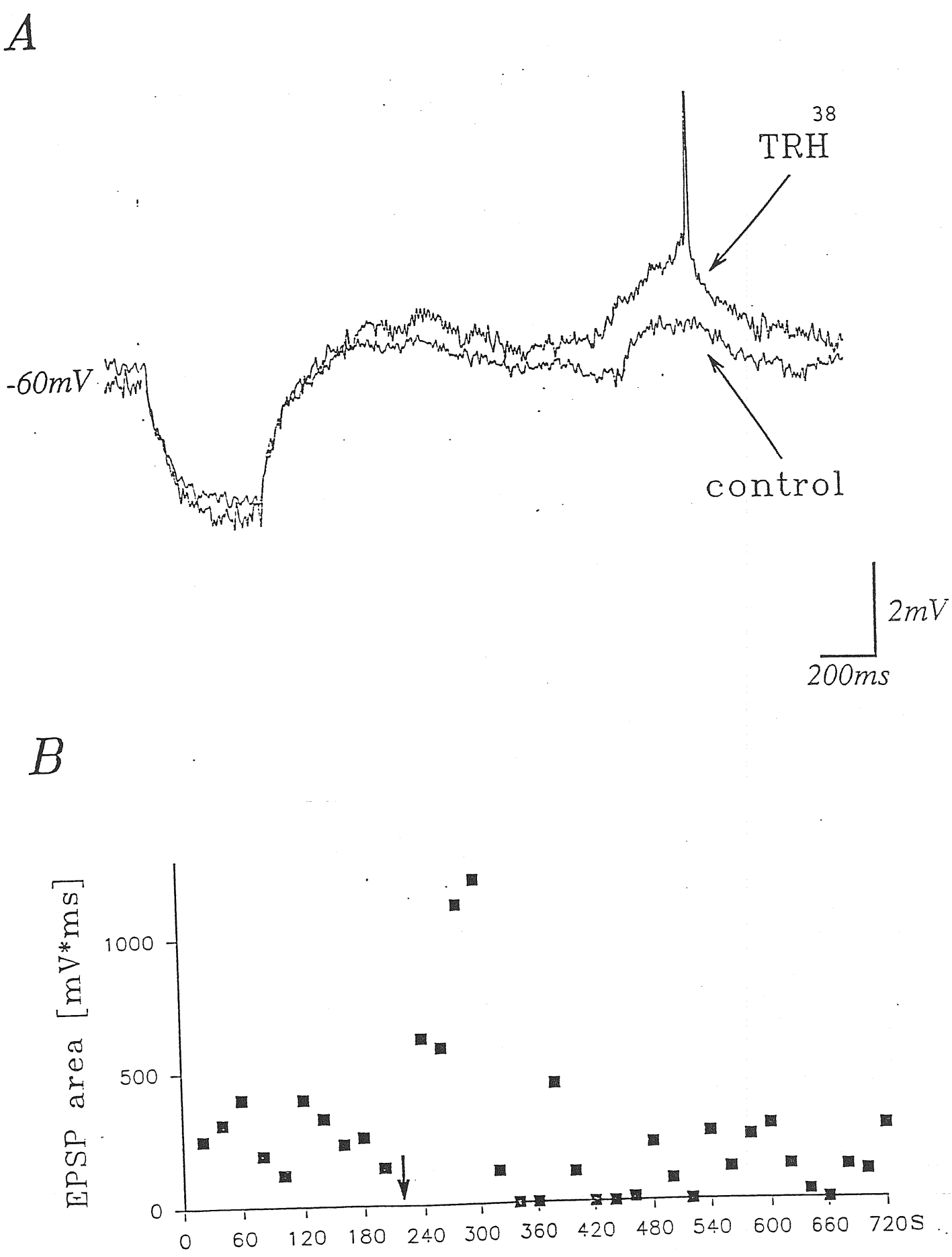


Fig. 9 Timecourse of the action of TRH (20 μM) on NMDA receptor-mediated EPSP. A: superimposed traces of 10 averaged events in control solution and of 4 averaged events after 100 s exposure to TRH: note that in TRH solution spiking occurs at the peak of enhanced EPSP whereas amplitude of hyperpolarizing electrotonic potential elicited before EPSP is not changed (indicating same value for input resistance). B: changes in EPSP area are plotted against time to show the transient change in the responses after application of TRH (arrow).

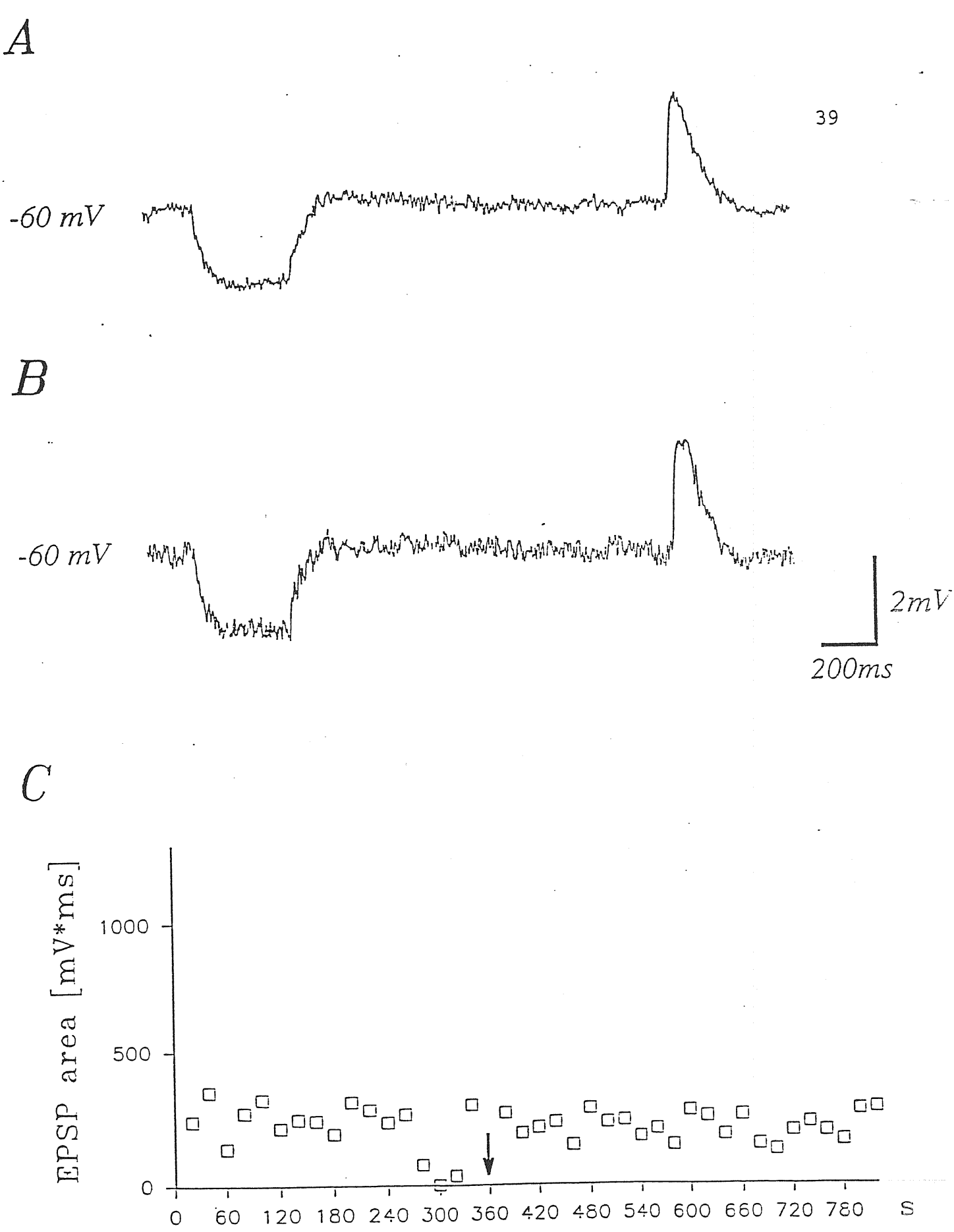


Fig. 10 AMPA/kainate receptor-mediated EPSPs in the presence of TRH (20 μM)
A: average trace of 17 control responses comprising hyperpolarizing electrotonic potential and EPSP; **B**: average trace of 4 responses after 100 s exposure to TRH. Note that there is no change in either hyperpolarizing electrotonic potential or EPSP. **C**: values of EPSP areas are plotted against time to show that TRH has no effect on these

which CNQX was replaced by CPP (10 µM) or D-aminophosphonovaleric acid (APV, 50 µM) in order to block NMDA receptors and to unveil AMPA/kainate receptor mediated EPSPs. The average amplitude, area and t_d of these EPSP. were 2.8±0.6 mV, 264.4±54.7 mV*ms and 68.1±7.8 ms, reflecting their faster timecourse. In the presence of TRH (10 µM) peak amplitude, area and t_d values remained unchanged during the first 2 min period of peptide administration (90.8±8.5, 93.0±10.3 and 101.5±20.6%, respectively) as well as during the subsequent 7 min (96.83±5.73, 92.5±5.42 and 91.66±7.49%). Fig. 10 A,B shows an example of traces obtained in control (average of 17 responses) or in TRH solution (average of 4 responses, thus yielding a much noisier record) with no obvious difference in the EPSP amplitude (2.57 *vs.* 2.58 mV) or area (97% of control). The lack of effectiveness of TRH on AMPA/kainate receptor mediated EPSPs could not be ascribed to the influence of prior administration of TRH used to test the NMDA receptor sensitivity. In fact, on 2 cells TRH was similarly ineffective when first tested on AMPA/kainate receptor-mediated EPSPs.

2.2 SPONTANEOUS

Unlike electrically-evoked EPSPs, it was difficult to observe spontaneous glutamatergic EPSPs either in control (in the presence of the above mentioned AMPA or NMDA antagonist) or TRH solution: in only 3/15 neurones, after about 5 min TRH application, the occurrence and size of this spontaneous activity was increased for about 1 min only.

2.3 RESPONSES TO NMDA AND AMPA

In order to check if the potentiation of NMDA receptor mediated responses involved postsynaptic glutamate receptors, the effect of TRH on the glutamate agonists: NMDA and AMPA was investigated.

NMDA or AMPA applications

Results were obtained from 32 hippocampal neurons with a membrane potential of -61±1 mV and 69±6 MΩ input resistance at rest. In Fig. 11 (A and B) a record obtained in DCC mode from one of these cells can be observed: spontaneous spiking activity on top of synaptic events was superimposed with regular upward and downward deflections of membrane potential elicited by depolarizing and hyperpolarizing steps, respectively. Repeated bath applications of NMDA (5-15 μM) (usually ≤1 min duration) (Fig. 11 A) were delivered every 10-15 minutes in control ACSF. Fig. 11 A shows the responses to the first and the forth application: note that the amplitude remained unchanged.

The duration of application and the agonist concentration were adjusted in each cell to elicit a relatively small depolarization (11±3 mV). NMDA responses developed within a few seconds after the end of application and were characterized by augmentation of synaptic activity leading to depolarization and increase in spiking activity. Repolarization of the membrane potential was attained after about 4 minutes, and complete recovery to resting level activity in about 10 min. There was an apparent increase (about 20%) in the third and forth response with respect to the first one, but this phenomenon was not statistically significant (Fig. 12, open triangles).

Another set of experiments was performed on a different group of cells (n=5) using AMPA, a non-NMDA glutamate agonist (Fig. 11B).

AMPA (5-10 μM) was repeatedly applied following the same protocol used for NMDA. To elicit similar depolarizations (11.2±2.8 mV) the duration of applications was shorter (10-30 s) compared with the NMDA one, and the depolarizations produced were faster to develop and slower to recover to the same V_m. Usually, even after recovery of membrane potential, spiking activity persisted (indicating lowering of spike threshold) so further applications of AMPA were delivered after a longer wash (20-25 minutes in ACSF). Note that the amplitude of AMPA

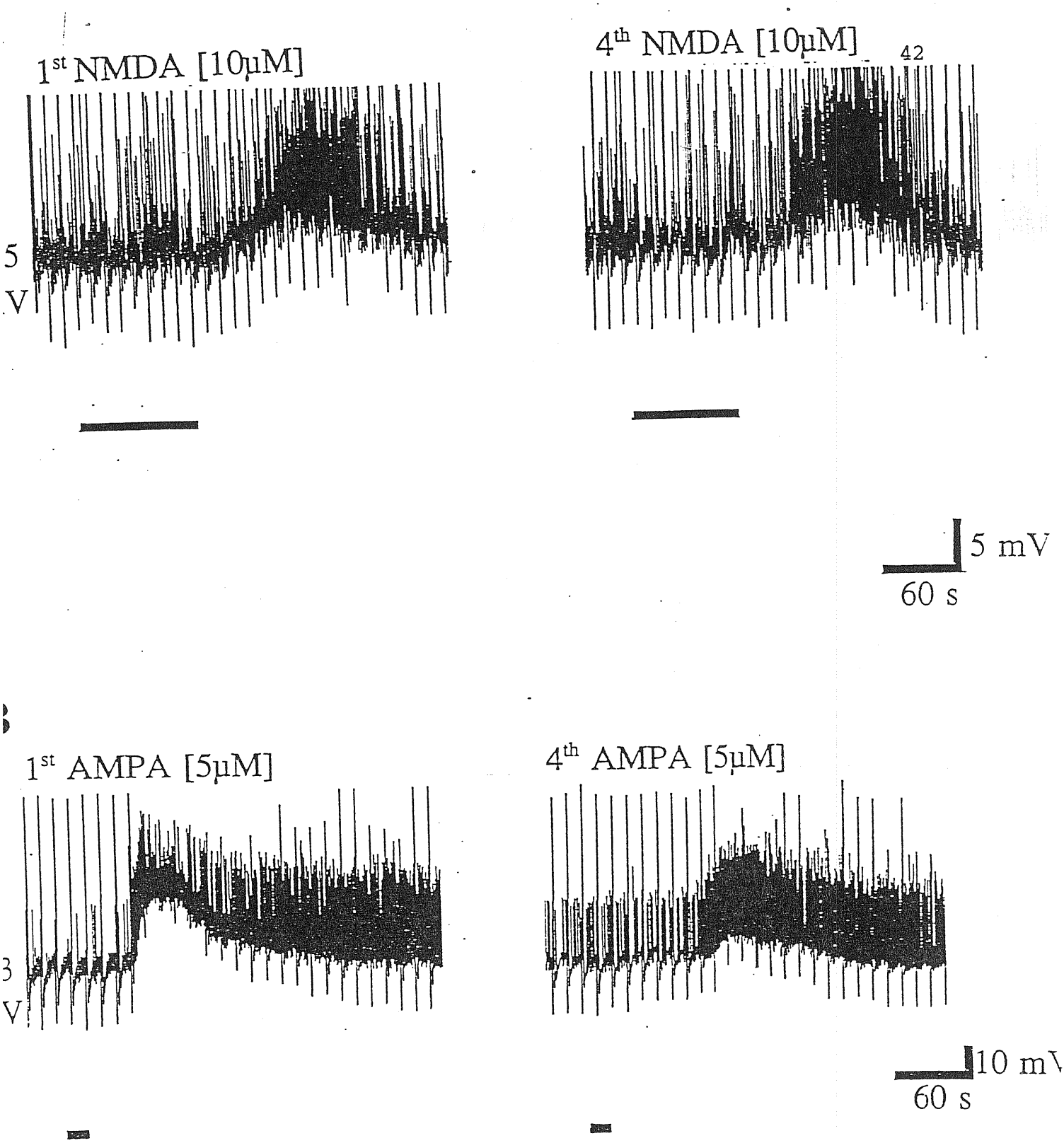


Fig. 11 Effect of repeated applications of glutamate agonists on CA1 hippocampal neurons. **A**: Chart record of membrane potential recording in control solution. NMDA was applied for the duration of the bar. Hyperpolarizing (400 ms) and depolarizing (350 ms) current pulses (0.15 and 0.3 nA, respectively) were regularly injected via the microelectrode at 0.1Hz. Time interval between applications was 10min. Responses were not significantly changed. Spikes truncated by frequency response of recorder.
B: Chart record of membrane potential recording in control solution. AMPA applications (see bar) elicited desensitized responses. AMPA was slower to wash, time interval between applications was 15 min.

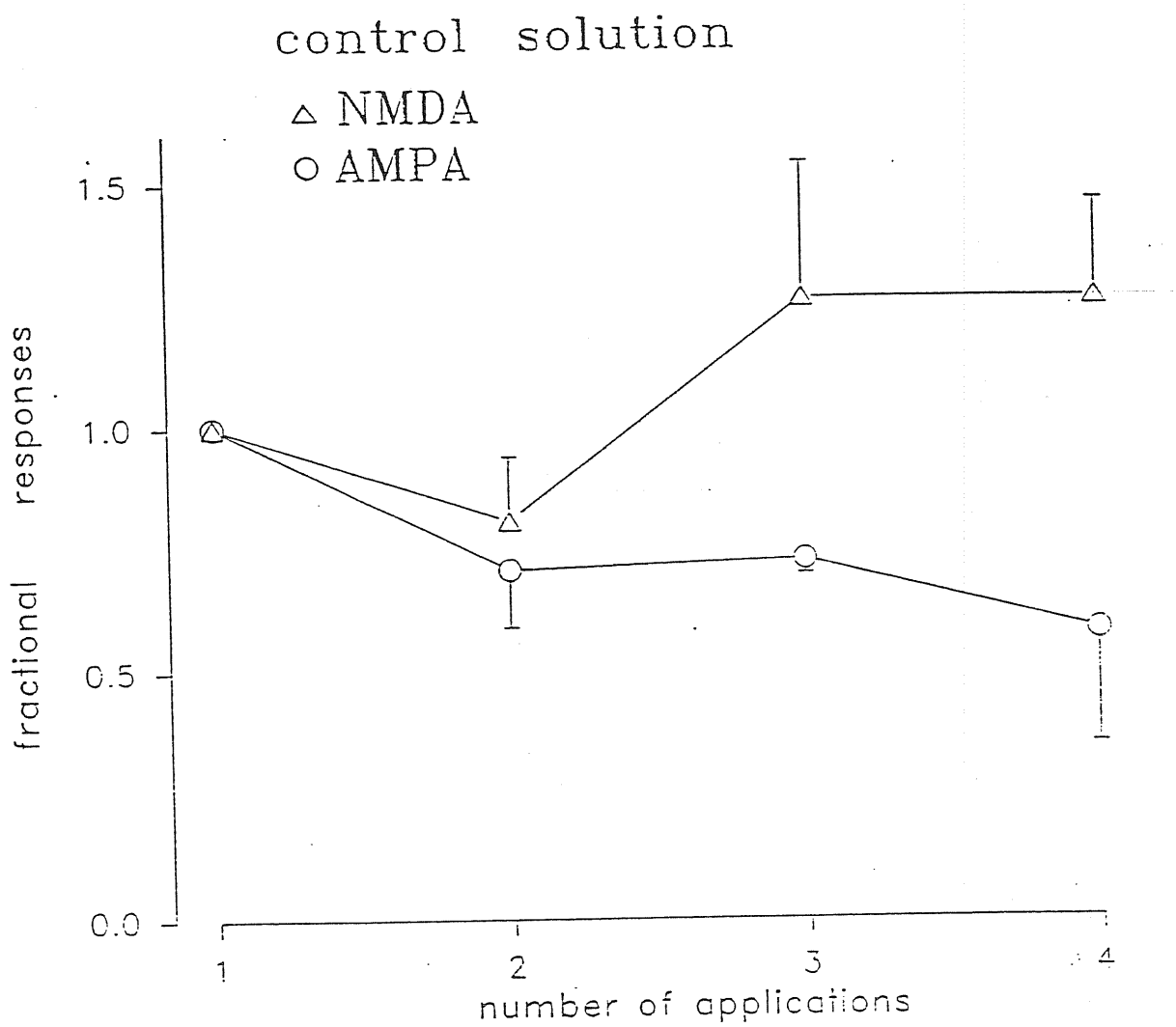


Fig. 12 Comparison between NMDA and AMPA responses amplitudes in control solution. NMDA (open triangles) and AMPA (open circles) responses amplitudes (ordinate) *vs* number of agonist applications (abscissa). Responses were normalized with respect to the depolarization amplitude of the first response.

responses was decreasing with further administrations (Fig. 12, open circles), a phenomenon due to strong desensitization of AMPA receptors (Trussel et al., 1993).

2.3.1 TRH effect on responses to NMDA and AMPA

During continuous application of TRH repeated doses of NMDA or AMPA were administered with the same duration and concentration used in control ACSF. TRH showed a modulatory effect leading to a potentiation of NMDA induced responses as indicated in Fig. 13 A, B.

Note that the onset of the depolarization elicited by NMDA was not significantly changed. On the other hand, the response was increased both in terms of amplitude and duration: in this example the depolarization itself was intense enough to block spiking activity. Between applications the cells were washed for at least 10-15 minutes in order to reattain the same V_m as before the NMDA application (Fig. 13 A).

This potentiation can be readily observed when responses to NMDA in control solution (open triangles of the plot in Fig. 13 B) are compared with NMDA responses in TRH solution (filled circles) which were normalized with respect to the last response before TRH application. One and two asterisks indicate $P<0.05$ and $P<0.02$ respectively.

In 8/10 neurons the first response (Fig. 13 B) in TRH solution was 4.7±1.5 times larger than its preceding control ($P<0.02$ for actual depolarization) although such a potentiation declined with the second responses being only 1.6±0.1 times larger ($P<0.05$). Further NMDA applications did not show a significant potentiation.

AMPA was applied to another set of neurons (n=5). The small increase in amplitude of AMPA responses in TRH solution was not statistically significant (see Fig. 14 A, B).

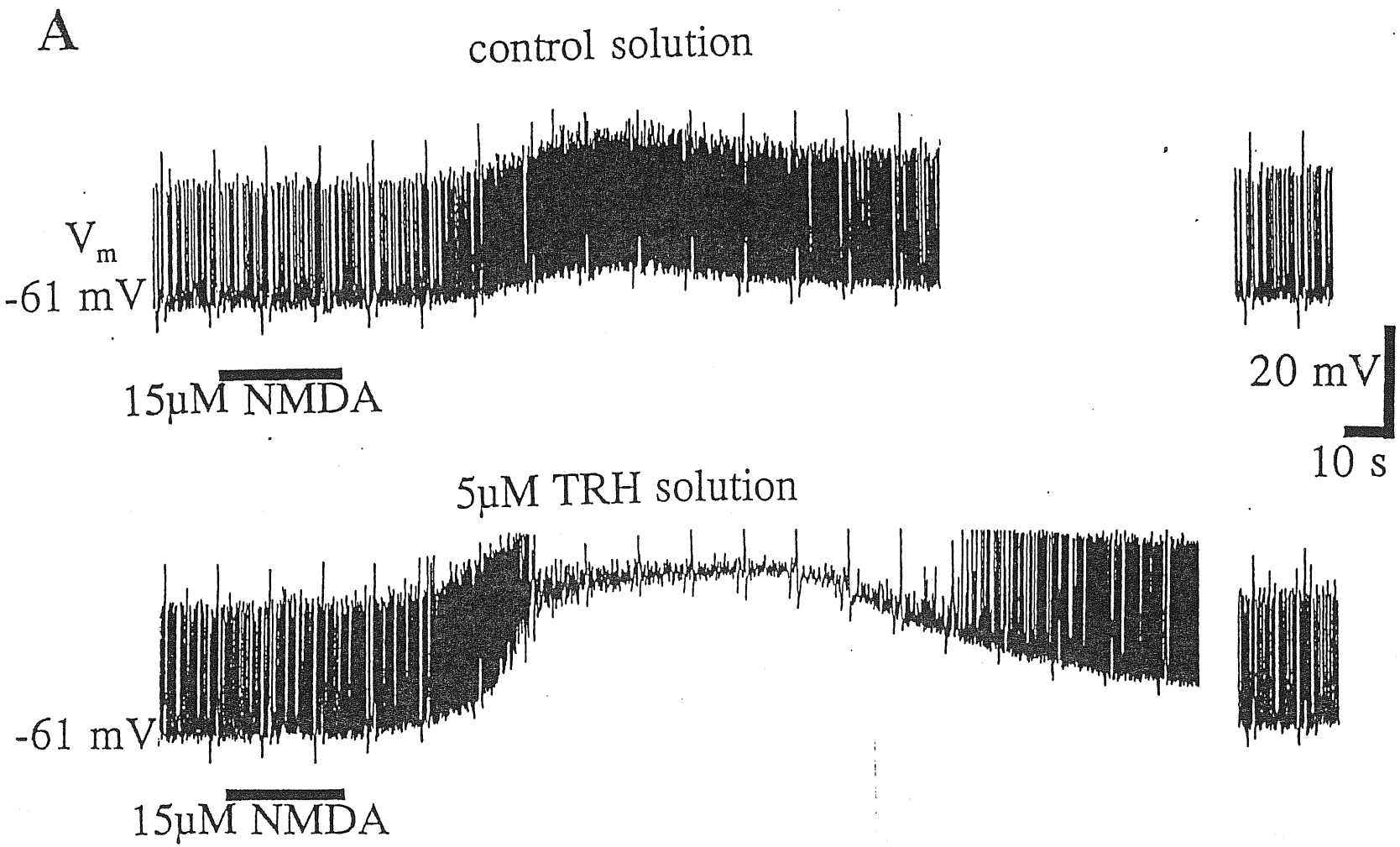


Fig. 13 A Effect of TRH on NMDA responses. Chart records of NMDA response in control solution (top trace) or in the continuous presence of TRH (bottom). The top trace shows the response produced by the fourth application of NMDA at resting membrane potential of -61 mV. The gap in the trace corresponded to about 10 min wash after which recovery was attained. The bottom trace shows that on the same cell at the same membrane potential, after 5 min exposure to TRH, the same application of NMDA elicited a much larger response. Full recovery was again obtained after 10 min (see trace after gap). Note increase of synaptic activity in TRH before NMDA application. (for current pulses see Fig. 11)

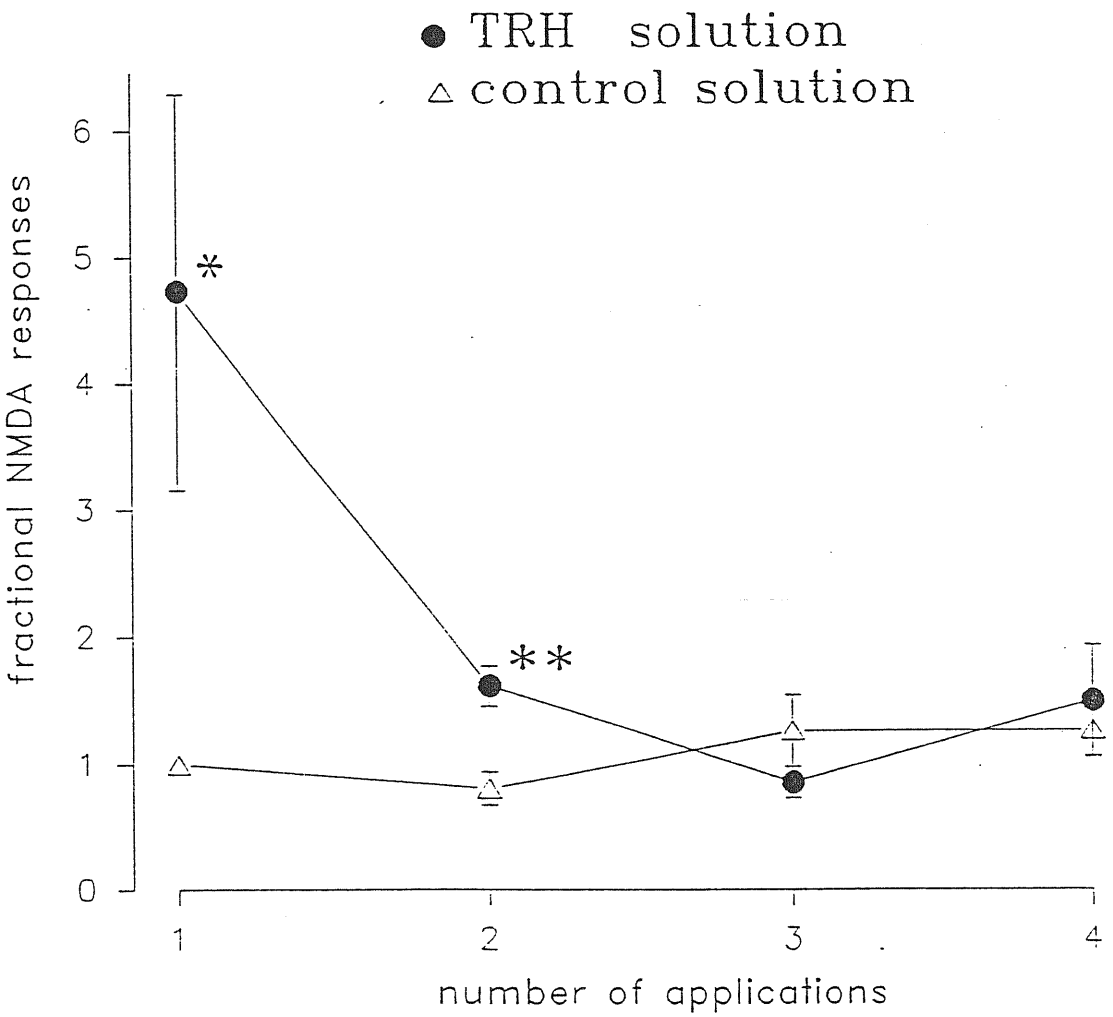
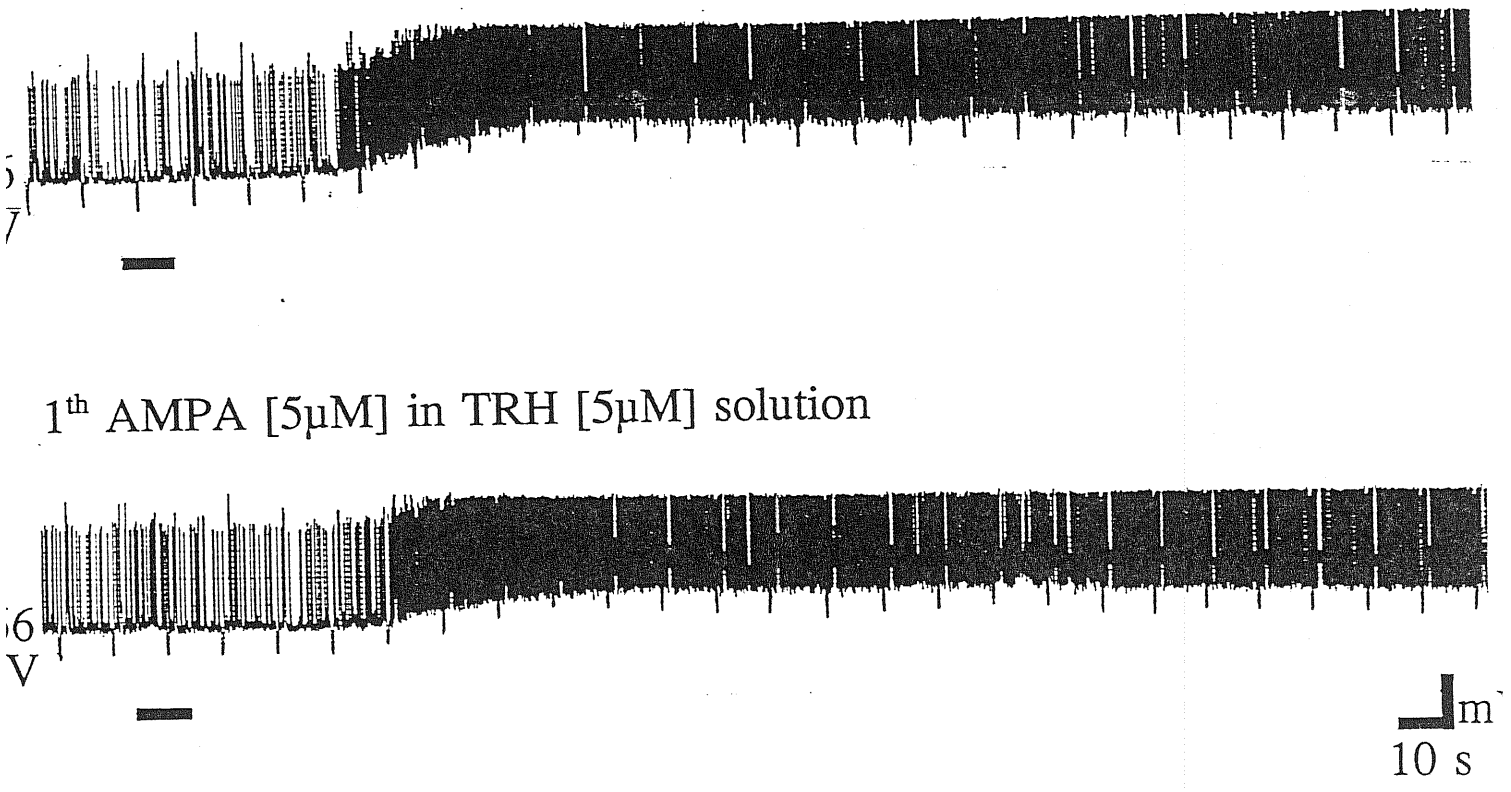


Fig. 13 B Effect of TRH on repetitive NMDA applications. Comparison between NMDA responses elicited in control solution (open triangles) and after 5 min in 5 µM TRH (filled circles). Control responses were normalized as in Fig.12 legend, while TRH responses were normalized with respect to the last response in control solution. Asterisks indicate that the difference between responses in the presence of TRH and their respective controls was statistically significant (*=P<0.02 and **=P<0.05, respectively when tested with the paired *t*-test on original data).


4th AMPA [5μM] in control solution
1th AMPA [5μM] in TRH [5μM] solution
10 s

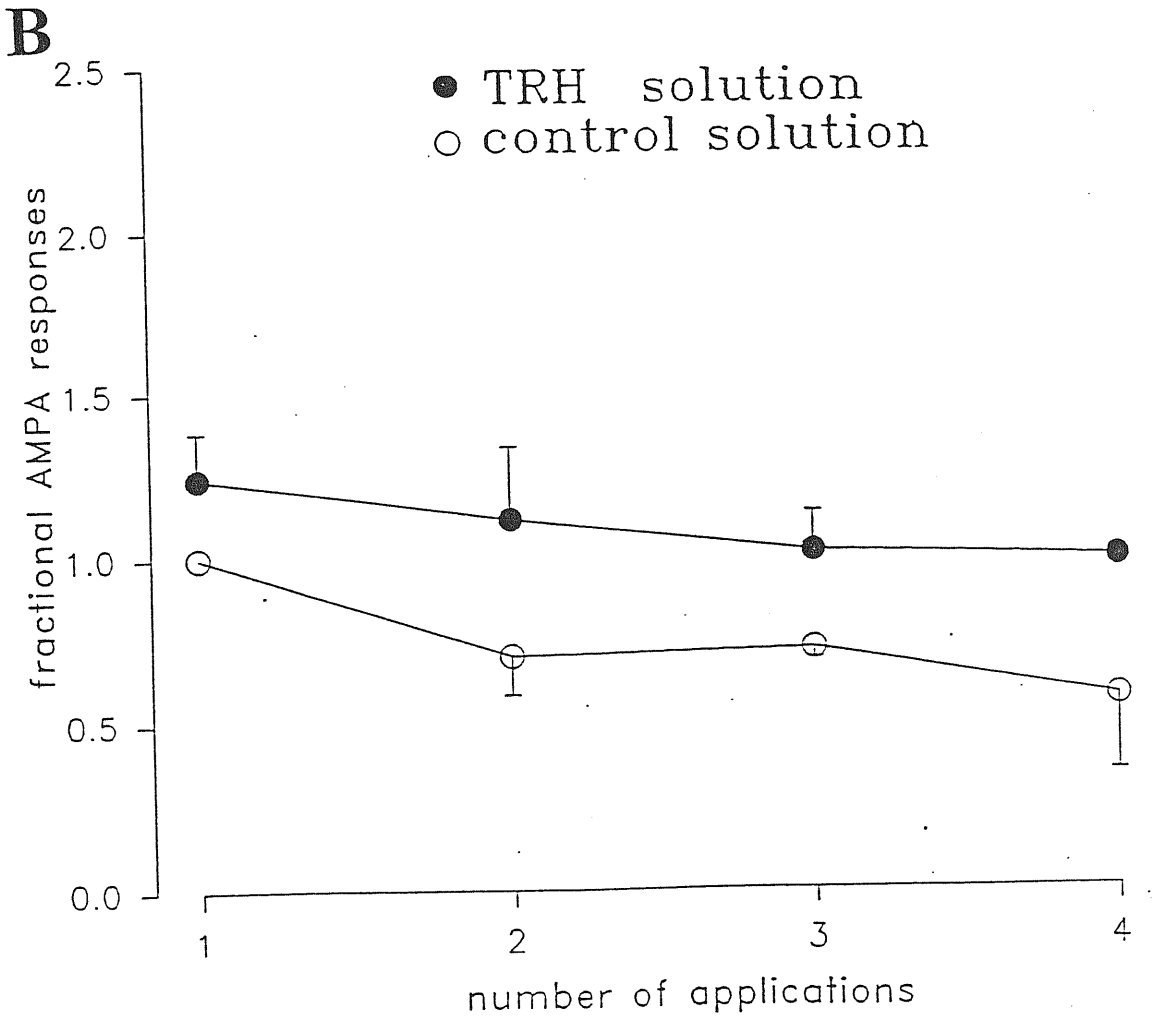

B
TRH solution
control solution
fractional AMPA responses
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
number of applications

Fig. 14 Effect of TRH on AMPA responses. **A**: Recording of AMPA response in control solution (top) or in the continuous presence of TRH (bottom). The two responses were not significantly different. **B**: Plot showing a comparison between AMPA responses elicited in control solution (open circles) and after TRH application (filled circles). Normalization as in Fig. 12.

2.3.2 Effect of TRH in tetrodotoxin (TTX) solution

In order to rule out TRH-induced presynaptic effects due to impulse dependent activity, experiments were conducted in solutions containing TTX (0.5-1 μM), a toxin extracted from the puffer fish, which blocks voltage activated Na^+channels. In this solution larger doses of NMDA (30 mM) were required to elicit responses (9.1±1.4 mV) matching the amplitude of those observed in control solution. As shown in Fig. 15 (open squares), these responses remained stable after repeated application.

In 5/11 cells bathed in TRH (5 μM) plus TTX solution the action of NMDA was initially potentiated (1.8±0.1 times larger than its control in TTX medium; $P<0.05$) (Fig. 15, filled squares) even though the subsequent responses were not significantly enhanced. In Fig. 16 (upper traces) some aspects of the potentiation of the first response to NMDA can be observed. In this example upward deflections were partial Ca^{2+}spikes induced by depolarizing current steps; downward deflections were either hyperpolarizing current steps (the larger ones) or afterhyperpolarizing potentials induced by Ca^{2+}-dependent K^+currents following the Ca^{2+}spikes. On top of the depolarization evoked by NMDA some membrane oscillations can also be observed. The slow membrane depolarization produced by the control application of NMDA was associated with an *apparent* increase in input resistance due to a characteristic of the channels involved (compare traces 1 and 2). The NMDA channel is both a ligand-gated and voltage dependent channel in the presence of extracellular Mg^{2+}(Novak et al.,1984): when the agonist activates it, Mg^{++} starts blocking it as long as the membrane potential is more negative than about -55 mV. The NMDA-induced depolarization is thus accompained by an apparent resistance increase, when measured with negative current steps, simply because the electrotonic hyperpolarizating potentials reach V_m values at which the Mg^{2+} block becomes operative. In the presence of TRH, at the same membrane potential, the amplitude of the hyperpolarizing potential was actually smaller, not

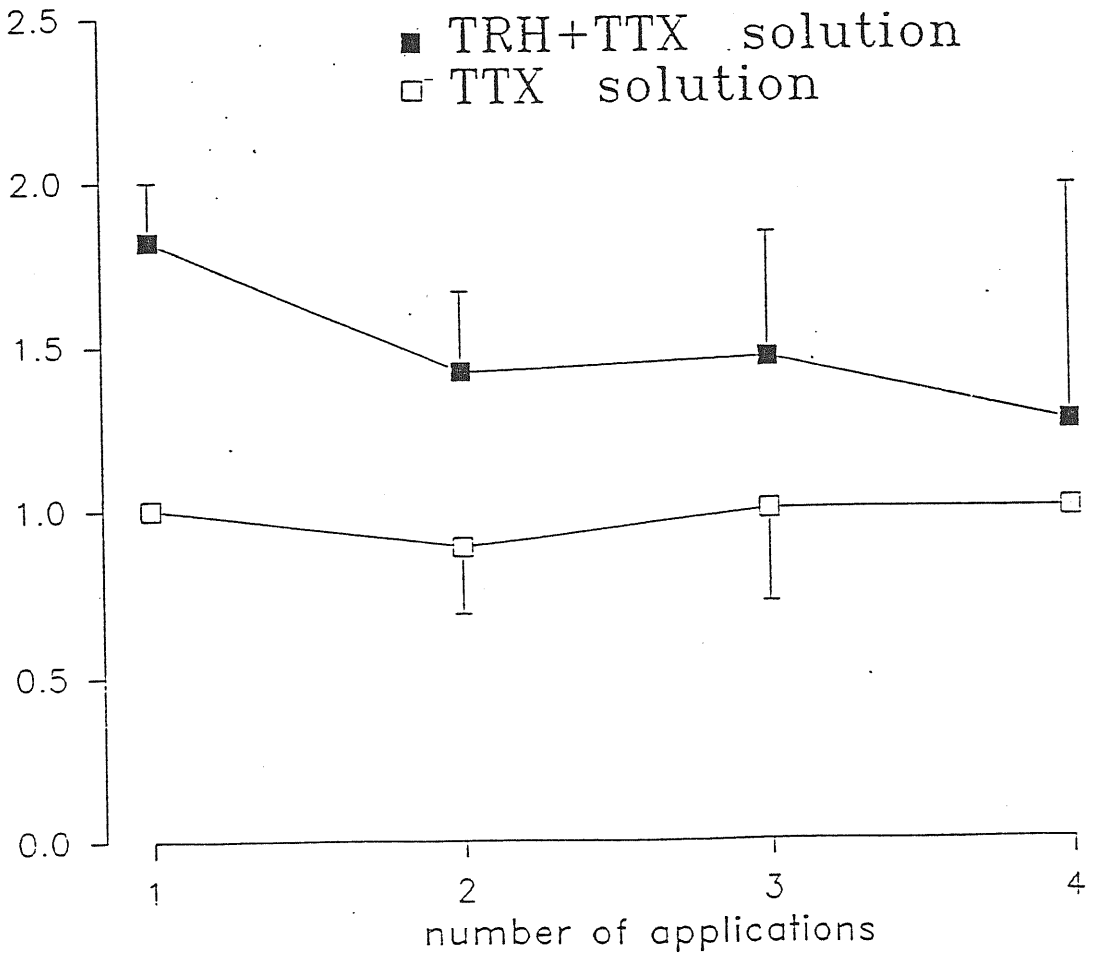


Fig. 15 Effect of TRH on NMDA responses in TTX solution. Comparison between NMDA responses in TTX solution (open squares) and in TRH added TTX solution. Responses were normalized as in Fig 12.

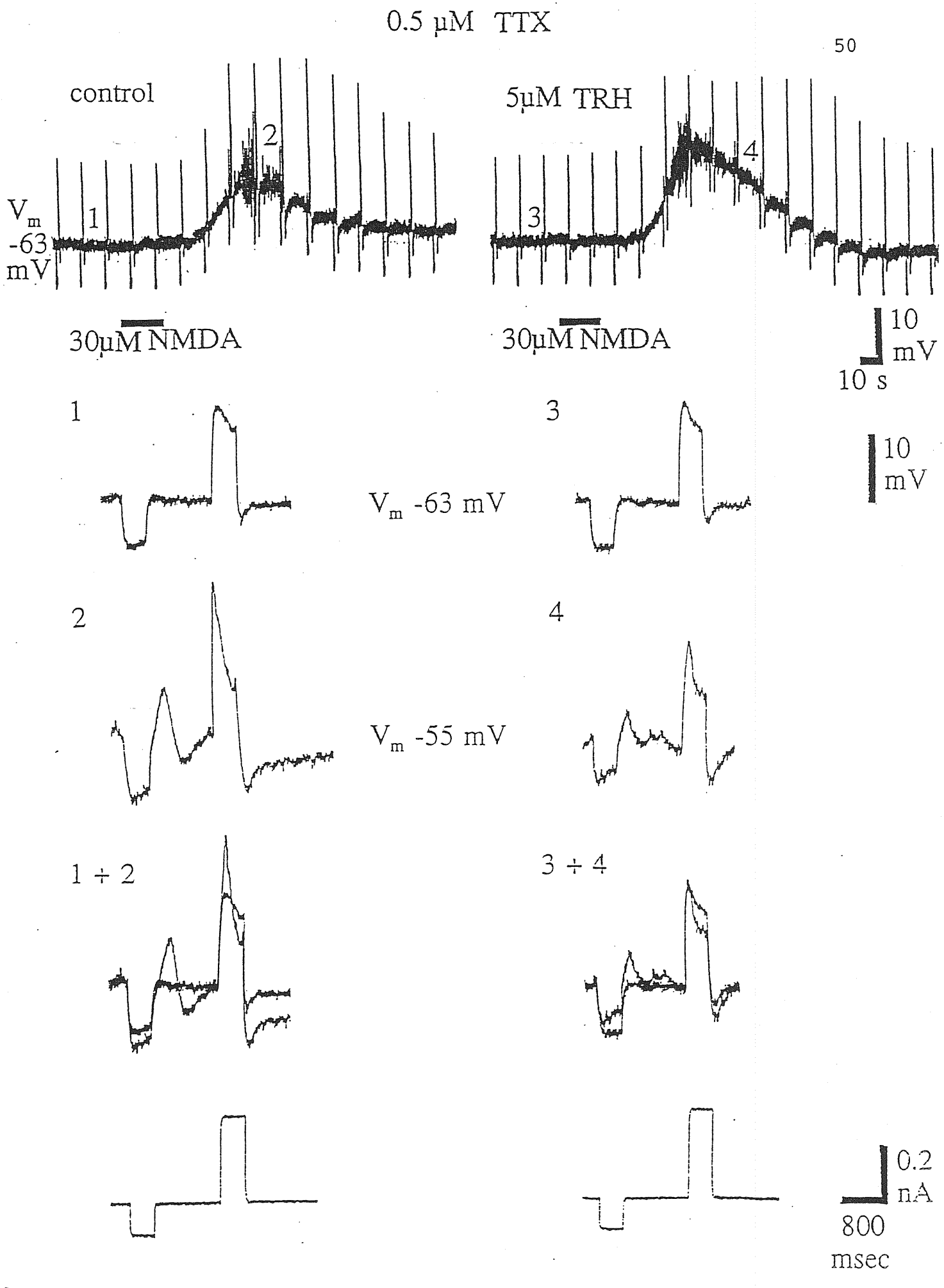

0.5 µM TTX
control
5µM TRH
V_m
-63
mV
1
2
3
4
30µM NMDA
30µM NMDA
10
mV
10 s
1
3
10
mV
V_m -63 mV
2
4
V_m -55 mV
1 + 2
3 + 4
0.2
nA
800
msec

Fig. 16 Effect of TRH on NMDA responses in TTX solution. Representative tracings from the same cell with -63 mV membrane at rest. TTX (0.5 μM) was present throughout. Top records show (on a slow time course) the potentiation of the response to NMDA (see bar for the time of application) after 5 min continuous exposure to TRH (5 μM). Hyperpolarizing and depolarizing current pulses (shown on a faster timebase in the bottom row) were regularly injected through the pipette. Middle rows present expanded, faster records of hyperpolarizing and depolarizing electrotonic potentials corresponding to those numbered from 1 to 4 in the top slow tracings. To aid comparison fast timebase responses before and during application of NMDA were superimposed (see record labelled 1+2 and 3+4).

larger, than before NMDA (Fig. 16 traces 3 and 4), a result indicative of an input resistance decrease. Consistent with this phenomenon was the attenuation of the depolarizing electrotonic potential and the partial spike.

3. MODULATION BY TRH OF GABAERGIC ACTIVITY

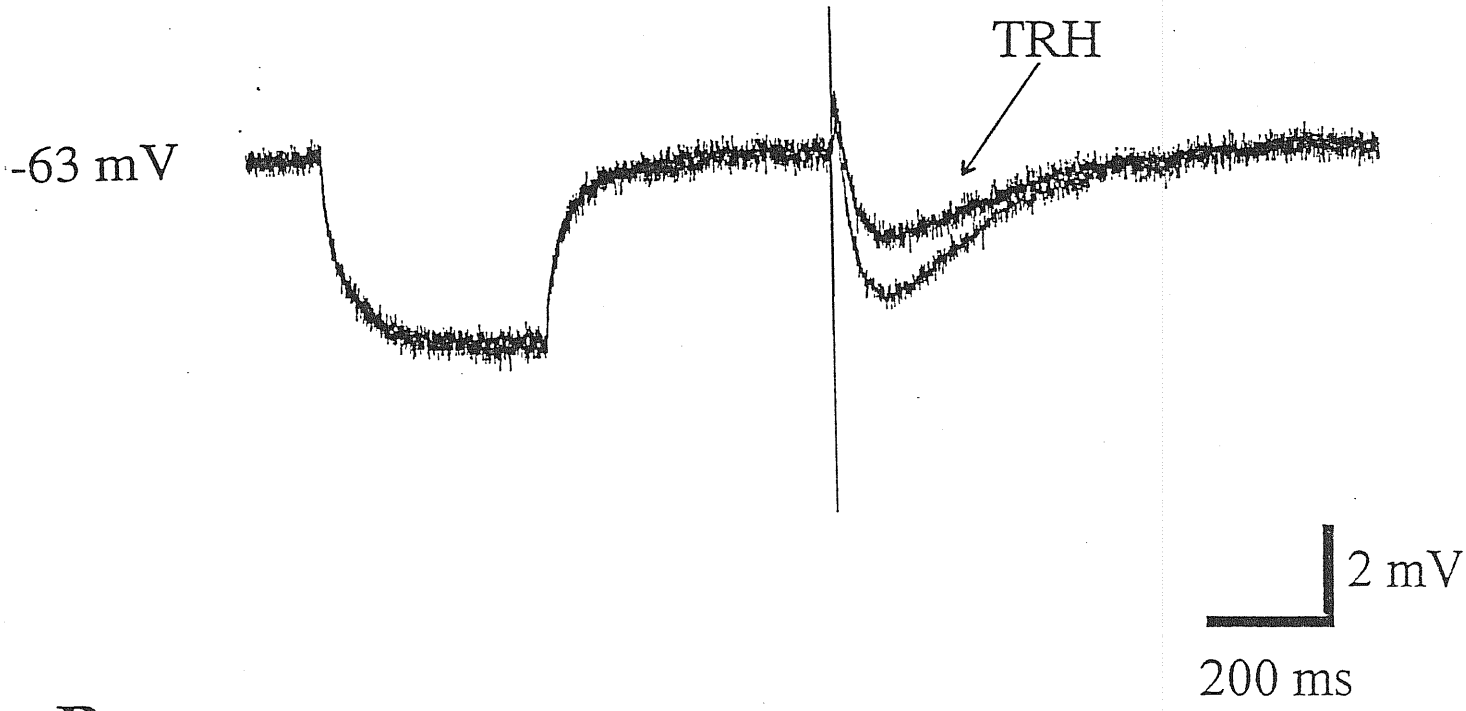
3.1 EVOKED POTENTIALS

The database of the present experiments comprises 37 CA1 pyramidal neurones (-68.9±1.2 mV Vm). TRH (10 µM; applied for up to 15 min) also in this population sample did not significantly change either the resting input resistance or the Vm of these cells. Such a lack of effect is exemplified by the superimposed averaged tracings of Fig. 17 A where the baseline level and the size of the hyperpolarizing electrotonic potential are identical before or after applying TRH.

3.1.1 $GABA_A$ receptor mediated IPSPs

Fast IPSPs, obtained with 2M K-methylsulphate and elicited by electrical stimulation of the stratum LM were identified as mediated by $GABA_A$ receptors since they were fully blocked by adding bicuculline (20 µM) to the glutamate, blocked by kynurenic acid or CNQX and CPP, and $GABA_B$ receptor(blocked by CGP 35348) blocking solution (see methods). Fig. 17 A shows an example of an averaged (10 single traces) trace recorded from a neurone at -63 Vm. In control solution (containing kynurenic acid and CGP 35348) the IPSP had a peak amplitude of 4.7 mV measured at 90 ms from the stimulus artefact and was preceded by a small (0.6 mV) residual EPSP. The same type of trace obtained in the presence of TRH (after 15 min exposure) is superimposed to aid comparison. The only detectable action of TRH was a 34 % fall in the peak amplitude of the IPSP without apparent shift in its timecourse. On a sample of five cells (at -64±1 mV rmp) the IPSP peak amplitude was 2.3±0.4 mV in ACSF and was reduced to 75±8 % of its control in the presence of TRH (P=0.04). This depressant effect developed within the first 2 min

A


TRH
-63 mV
2 mV
200 ms

B

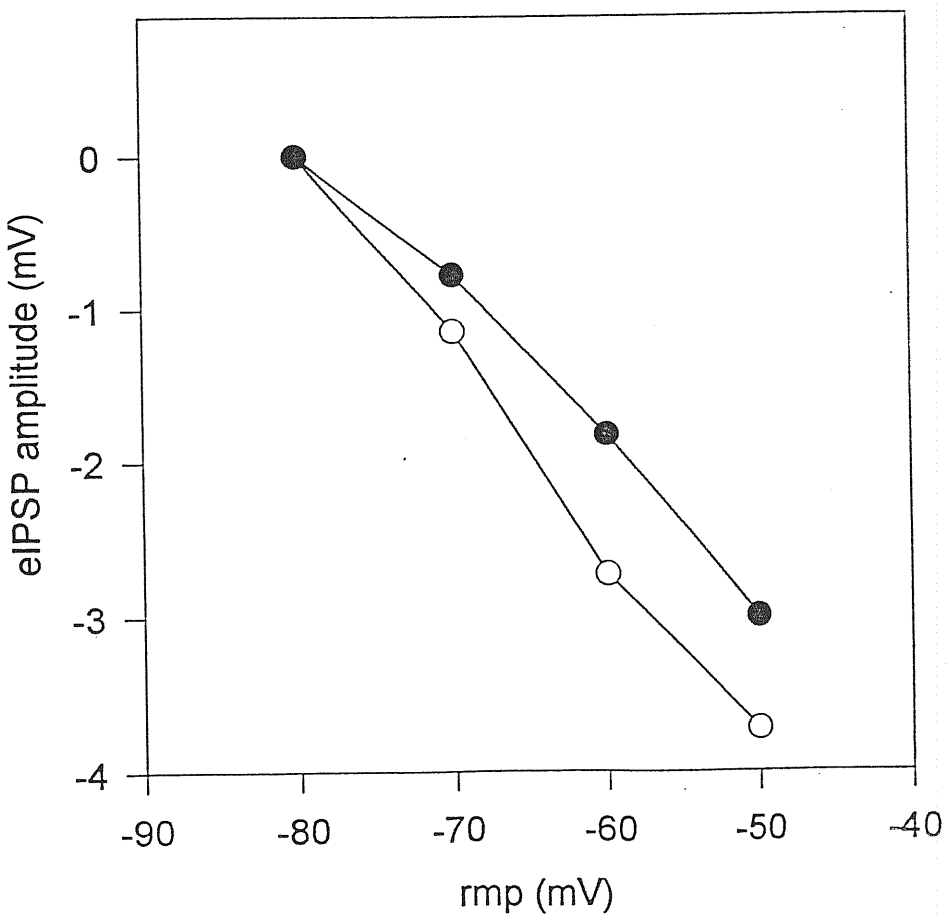

0
-1
-2
-3
-4
eIPSP amplitude (mV)
-90
-80
-70
-60
-50
-40
rmp (mV)

Fig. 17 Effect of TRH (10μM) on $GABA_A$ receptor mediated IPSPs evoked from Stratum Radiatum. **A**: average trace of 10 responses recorded in control solution, the fast $GABA_A$ receptor mediated was developing just after stimulation (see stimulus artefact). Average trace obtained in the same way in the presence of TRH (10μM) was superinposed to aid comparison (indicated by the arrow). In TRH the peak amplitude was reduced to 66% of control. Note that the input resistance remained unchanged. **B**: Plot representing the effect of TRH at different Vm in the same cell. The peak amplitude in control (open circles) and in TRH (close circles) plotted against changing Vm. Note the similar effect exerted by TRH at different Vm.

of TRH application, did not appreciably fade over about 5-7 min and was reversible after sustained washout (>30 min). Fig. 17 B (same cell of Fig. 17 A) shows the effect of changing membrane potential on the IPSP amplitude in control solution (open circles) or in the presence of TRH (filled circles). Note that in the presence of TRH there was a similar depression of the IPSP at the various rmp levels with no apparent change in the IPSP reversal potential which was -83 mV before and after application of TRH. On a sample of 5 cells there was no statistically significant change in IPSP reversal by TRH (-76.8±6.5 mV and -78.2±5.5 mV in control and TRH solution, respectively).

$GABA_A$ receptor mediated IPSPs were also evoked by electrical stimulation of GABAergic interneurones of the SP area (Alger, 1984) using again the same glutamate and $GABA_B$ receptor blockers as above. At an average Vm of -60.0±1.4 mV (n=4) TRH reduced the peak amplitude of these IPSPs from -4.24±0.87 to -1.82±0.92 ($P<0.05$) which corresponds to a 57% decrease with no significant change in IPSP reversals.

3.2.1 $GABA_B$ receptor mediated IPSPs

Single pulses or trains applied to the LM layer in the presence of kynurenic acid and bicuculline did not elicit synaptic potentials. In order to assess the action of TRH on pharmacologically-isolated $GABA_B$ receptor mediated IPSPs were recorded with 3 M KCl or 2M K-methylsulphate and the stimulating electrodes were thus moved to the SP layer in the attempt to activate directly inhibitory GABAergic interneurones which should generate $GABA_B$ receptor mediated responses (Alger, 1984). Fig. 18 A shows an example of average (10 single traces) IPSPs (induced by a single pulse applied to the SP area) recorded from a pyramidal cell at -63 mV rmp and characterized by shallow amplitude (0.6 mV) and a slow timecourse (460 ms time to peak). As these slow IPSPs were fully blocked by CGP 35348 (1 mM), they were identified as mediated by $GABA_B$ receptors. In the presence of TRH the synaptic responses were unchanged as indicated by

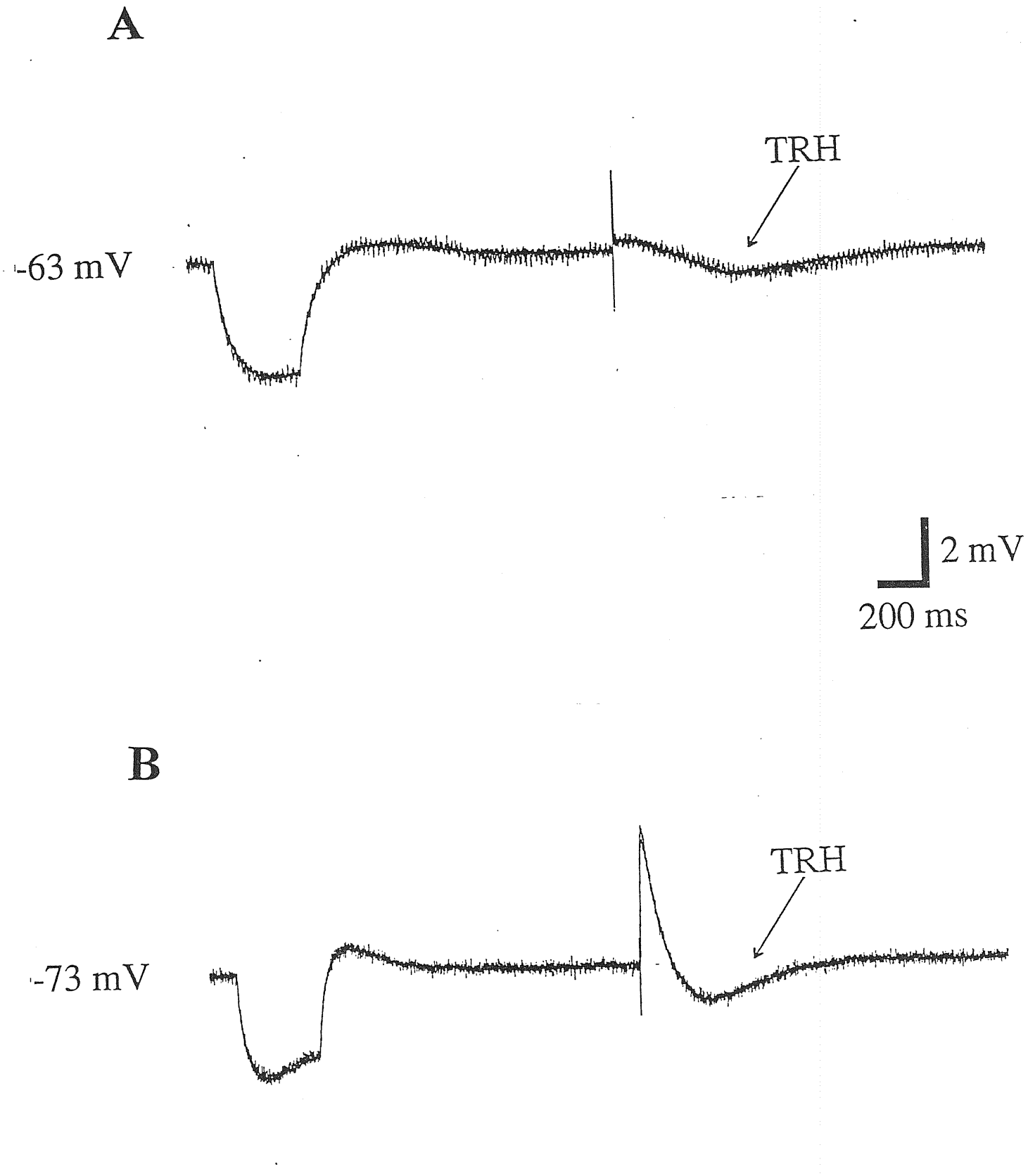


Fig. 18 Effect of TRH on $GABA_B$ receptor mediated IPSPs evoked from Stratum Pyramidale. **A**: overlapped averaged traces (10 single) traces recorded in control solution and in the presence of TRH (arrow). **B**: overlapped traces as before, AMPA receptor mediated EPSP was left. No change in the amplitude of the response can be detected. Traces recorded at a more hyperpolarized potential, note the developing of the inward rectifyer, to keep Vm far from firing threshold. Rm kept unchanged.

the exactly superimposed records. This result was borne out by similar observations on 6 cells in which slow IPSPs had an average peak amplitude of 1.05±0.11 mV at -71.6±1.5mV, and remained unaltered following TRH application (97±6 %). A more effective activation of local GABAergic cells to enhance slow IPSPs was attempted by avoiding blockade of the excitatory glutamatergic inputs impinging upon inhbitory neurones. For this purpose the glutamatergic AMPA receptor mediated EPSP was preserved while blocking the slow NMDA receptor mediated EPSP (which might have overlapped the slow IPSP due to its slow kinetic) with 10 µM CPP (bicuculline was also present throughout to eliminate fast IPSPs). In the example of Fig. 18 B the evoked synaptic response was thus biphasic with a large fast EPSP (3.8 mV amplitude and 20 ms time to peak) which was followed by a slow, CGP 35348-sensitive IPSP (0.9 mV amplitude and 260 ms time to peak). This cell was kept at -73 mV membrane potential to maintain the EPSPs considerably below spike threshold (-55 to -58 mV). The negative resting potential was also indicated by the appearance of a sag in the averaged electrotonic potential as the membrane potential entered a region of inward rectification (Halliwell and Adams, 1983). Even when the fast glutamatergic input was preserved there was no effect of TRH on the slow IPSP as shown by the fully overlapping traces before and after peptide application. Averaged responses obtained in TRH solution were the same as the control ones (105±2 %; n=3). In order to find out if the lack of sensitivity of slow IPSPs to TRH was a feature of this particular GABAergic input, namely a feature of the circuit underlying this response, further tests were carried out on the slow IPSPs evoked by SLM stimulation. The inability to elicit pharmacologically-isolated slow IPSPs with this type of stimulation (see above) required preserving the AMPA receptor mediated EPSP to drive the slow IPSP. Fig. 19 shows an example of these recordings obtained at -73 mV membrane potential. Overlapping traces obtained before and after application of TRH show that the slow IPSP was reduced by 33 % while no effect on

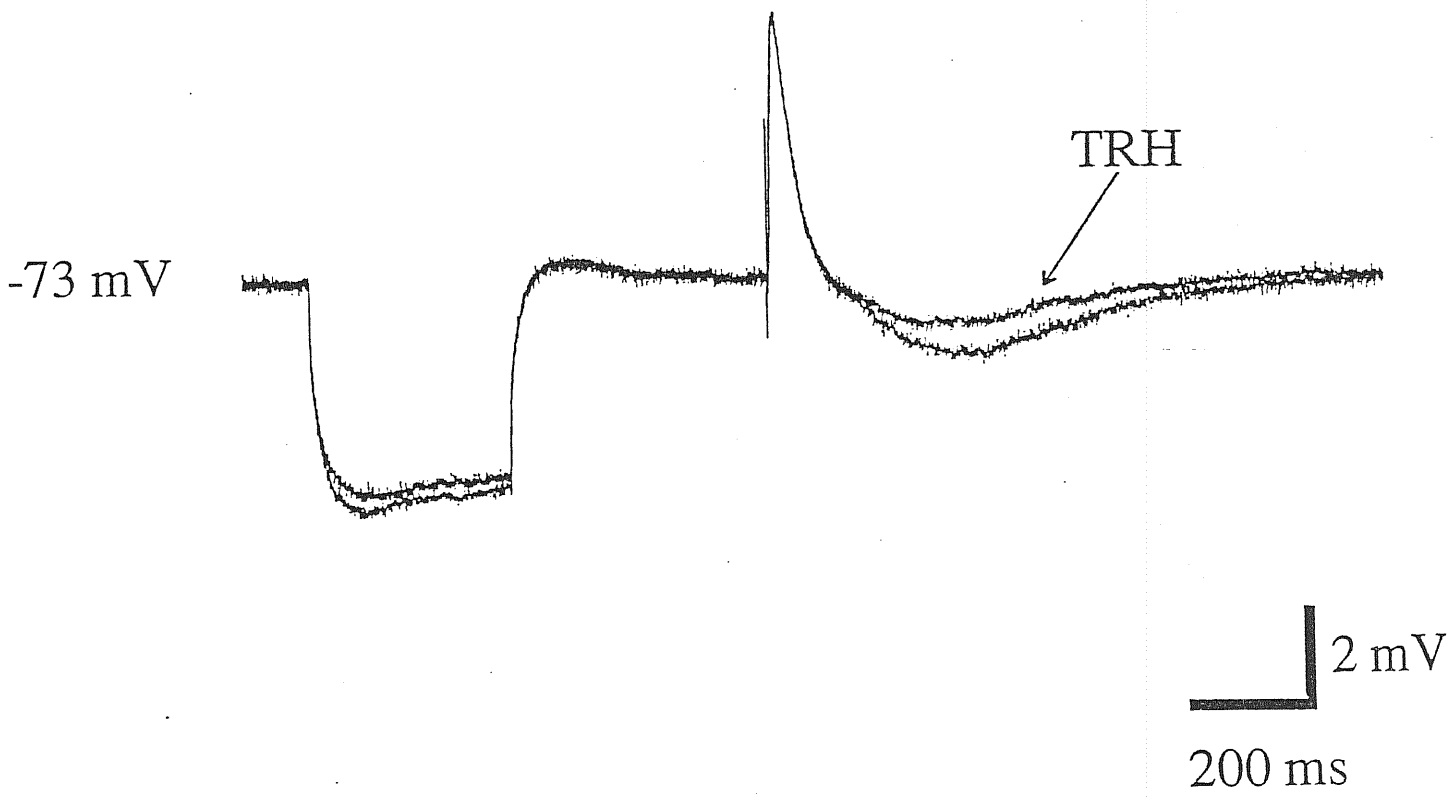


Fig. 19 Effect of TRH on $GABA_B$ receptor mediated IPSPs evoked from the Stratum Radiatum. AMPA receptor component was left. Overlapped traces in control and TRH solution (arrow). In TRH the peak amplitude was decreased to 67 % of control.

the fast EPSP was found. There was also a minor (5 %) increase in input resistance but this phenomenon was not significant as on average (n=5) the input resistance in the presence of TRH was 97±2 % of control. On 5 cells TRH reduced the $GABA_B$ receptor mediated IPSPs to 79±6 % (P=0.05) of control while the amplitude of the fast EPSPs was not significantly changed (91±3 %).

3.2 SPONTANEOUS

Spontaneous $GABA_A$ receptor mediated IPSPs of CA1 cells recorded with K-methylsulphate filled microelectrodes were infrequently observed in control solution and, when present, they were of small amplitude: in three cells from which sufficient data could be collected their average amplitude (at -57±2 mV membrane potential) was 0.99±0.09 mV and their frequency was 0.14±0.05 Hz, after setting the detection threshold at 3 times the standard error of the baseline noise. In the presence of TRH neither the amplitude (1.21 ±0.03 mV) nor the frequency (0.19 ±0.05 Hz) of these events was significantly changed. These results should however be interpreted with caution since a significant number of synaptic events might have remained below detection level. On the other hand, in the case of recordings made with KCl filled microelectrodes, TRH elicited (after 5-10 minutes from the application onset) a sustained increase in spontaneous electrical activity (see, for example, Fig. 13 A and 14 A: tract of the record before NMDA application). This spontaneous activity probably comprised EPSPs plus reversed IPSPs (due to intracellular build up of Cl^-). The latter usually represent the largest conponent of such a spontaneous activity (Ropert et al., 1990). Probably TRH induced this effect through a combination of presynaptic phenomena (e.g. modulation of transmitter release) and postsynaptic effects (e.g. increase in the excitability of the postsynaptic membrane, even if undetected by input resistance measurements). The difference in the action of TRH recorded with KCl or K-methylsulphate electrodes might be due to the limited resolution of intracellular techniques on

respect to small synaptic potentials: when the equilibrium potential for chloride is changed, as in KCl filled elecrodes, the responses are increased in amplitude.

3.3 RESPONSES TO ISOGUVACINE AND BACLOFEN

It seemed of interest to test the sensitivity of GABA receptors to TRH: to this end responses elicited by activation of $GABA_A$ or $GABA_B$ receptors by selective agonists such as isoguvacine and baclofen were studied before and after applying TRH. In 4/6 neurones these experiments were performed in the presence of kynurenic acid to block effects indirectly caused via release of glutamate and in 4/6 neurones it was possible to test both agonists on the same cell. As shown by the example of Fig. 20 A, bath application of isoguvacine (10 µM) produced a depolarizing response which had average amplitude of 20.1±1.3 mV (at -72±1 mV rmp in 6 cells recorded with KCl-filled microelectrodes). The response was associated with a large fall in input resistance (63±5 %) and was fully reversible on washout. In the presence of TRH the isoguvacine-elicited depolarization (21.3±1.2 mV) and input resistance rise (59±5 %) were the same as in control solution (*cf.* Fig. 20 A). Similar observations were obtained when baclofen was used as an agonist as shown in Fig. 20 B. The baclofen-elicited slow hyperpolarization was 6.2±0.8 mV (n=4) in control solution *vs* 7.0±1.0 mV in the presence of TRH. The resistance fall due to baclofen was 37±2 % and remained essentially unchanged in the presence of TRH (42±3 %). One should note that this resistance drop comprised hyperpolarization-induced membrane rectification since returning the cell to resting potential by depolarizing current injection (DC test in Fig. 20 B) attenuated the resistance fall due to baclofen. After eliminating the contribution by network interneurones to the observed responses by bath-applied tetrodotoxin (1 µM) (Fig. 21 A and B), isoguvacine or baclofen mediated membrane potential changes were 13.0±2.7 mV and -6.0±0.5

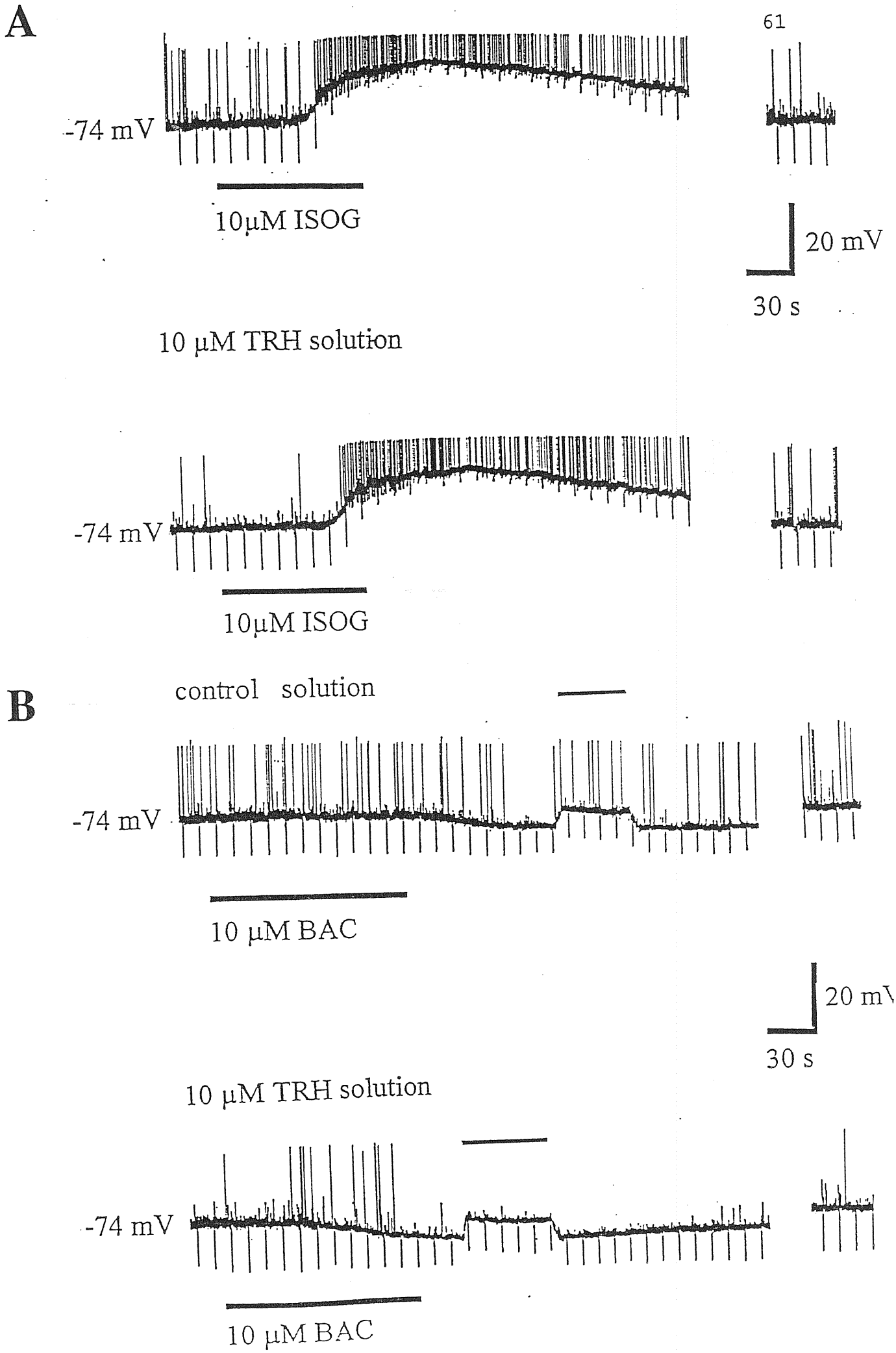

A
61
-74 mV
10μM ISOG
20 mV
30 s
10 μM TRH solution
-74 mV
10μM ISOG
B
control solution
-74 mV
10 μM BAC
20 mV
30 s
10 μM TRH solution
-74 mV
10 μM BAC

Fig. 20 Effect of TRH (10μM) on GABA agonists. A: chart record of membrane potential showing the responses to application of isoguvacine. No change in amplitude of the response was detected in TRH solution. B: after 1hwashout in the same cell baclofen was tested using the same protocol. As above TRH did not elicit any change. Downward and upward deflection were respectively pulses for monitoring the Rm and spikes truncated. The gap in the trace corresponded to 10-15 min wash after which recovery was attained. Recordings obtained with KCl filled pipette. Solid bars indicate drug application (lower ones) and DC test (upper ones).

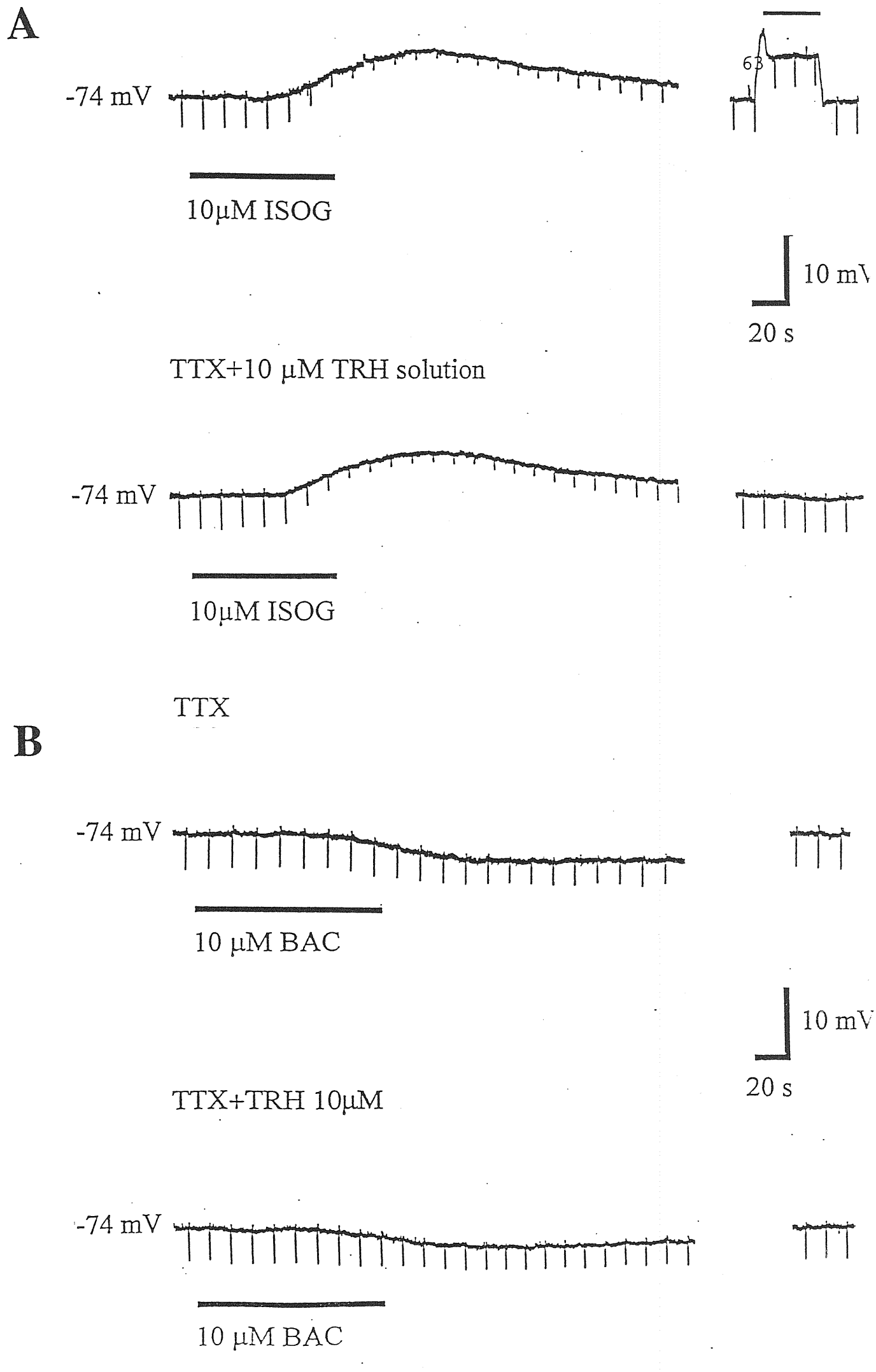

A
-74 mV
63
10μM ISOG
10 mV
20 s
TTX+10 μM TRH solution
-74 mV
10μM ISOG
TTX
B
-74 mV
10 μM BAC
10 mV
20 s
TTX+TRH 10μM
-74 mV
10 μM BAC

Fig. 21 Effect of TRH on GABA agonist in TTX (1µM). A: chart record of membrane potential showing responses to isoguvacine and in B: baclofen. No change in the amplitude of the responses was detected in either cases.After the a gap of 10-15 min Vm went back to previous level. Recordings obtained with KCl filled pipette. Solid bars indicate drug application (lower ones) and DC test (upper ones).

mV, respectively (Vm=66±1 mV; n=6). In the presence of TRH responses to isoguvacine or baclofen were 94±3 % and 101±7 % of their controls, respectively.

4. EFFECT OF TRH OF STP AND LTP

On a sample of 15 neurons with a Vm=-72±5 mV a train of high frequency stimuli (protocol described in the methods) were delivered in order to elicit STP and eventually LTP. In order to facilitate the developing of this phenomenon 10μM picrotoxin was continuously delivered via the bathing solution to depress $GABA_A$ mediated responses. Post synaptic potentials were recorded in DCC mode: a glutamatergic EPSP followed by a GABAergic IPSP, were evoked after extracellular stimulation of the Schaffer collateral fibers. The stimulus (11-30 V amplitude, 10-20μs duration) was delivered at 0.05 Hz in order not to produce further modulations.

4.1 POTENTIATION IN CONTROL SOLUTION

Preceding the delivery of the trains the EPSP peak amplitude, rising time and areas were 3.8±0.3 mV, 10.4±1.4 ms and 153±18 mV*ms respectively. In all cells tested (n=6) in control conditions trains caused an increased in EPSP area of 52±6% ($p<0.05$) and 56±17% ($p<0.05$) when measured after 20 or 50 min from the train, respectively.The Rm was not significantly changed being 103% and 100% (after 20 and 50 min) of control. The rise time was 80±12% and 116±26% of control at 20 and 50 min, respectively. In Fig. 22 trace averages representing the development of the potentiation can be seen. Each trace was obtained after averaging three subsequent single traces evoked 20 sec apart (0.05 Hz). The top trace represents an EPSP evoked before the high frequency stimulation. EPSPs measured over 10 min time were 3.6 mV, 14 ms and 166 mV*ms in amplitude, rise time and area, respectively.

The middle and bottom traces were recorded after 20 and 50 min from train stimulation respectively. After 20 min the peak amplitude and the area became 4.6 mV and 236 mV*ms with an potentiation of the response of 27% and 41% in the case of peak and area, respectively. The

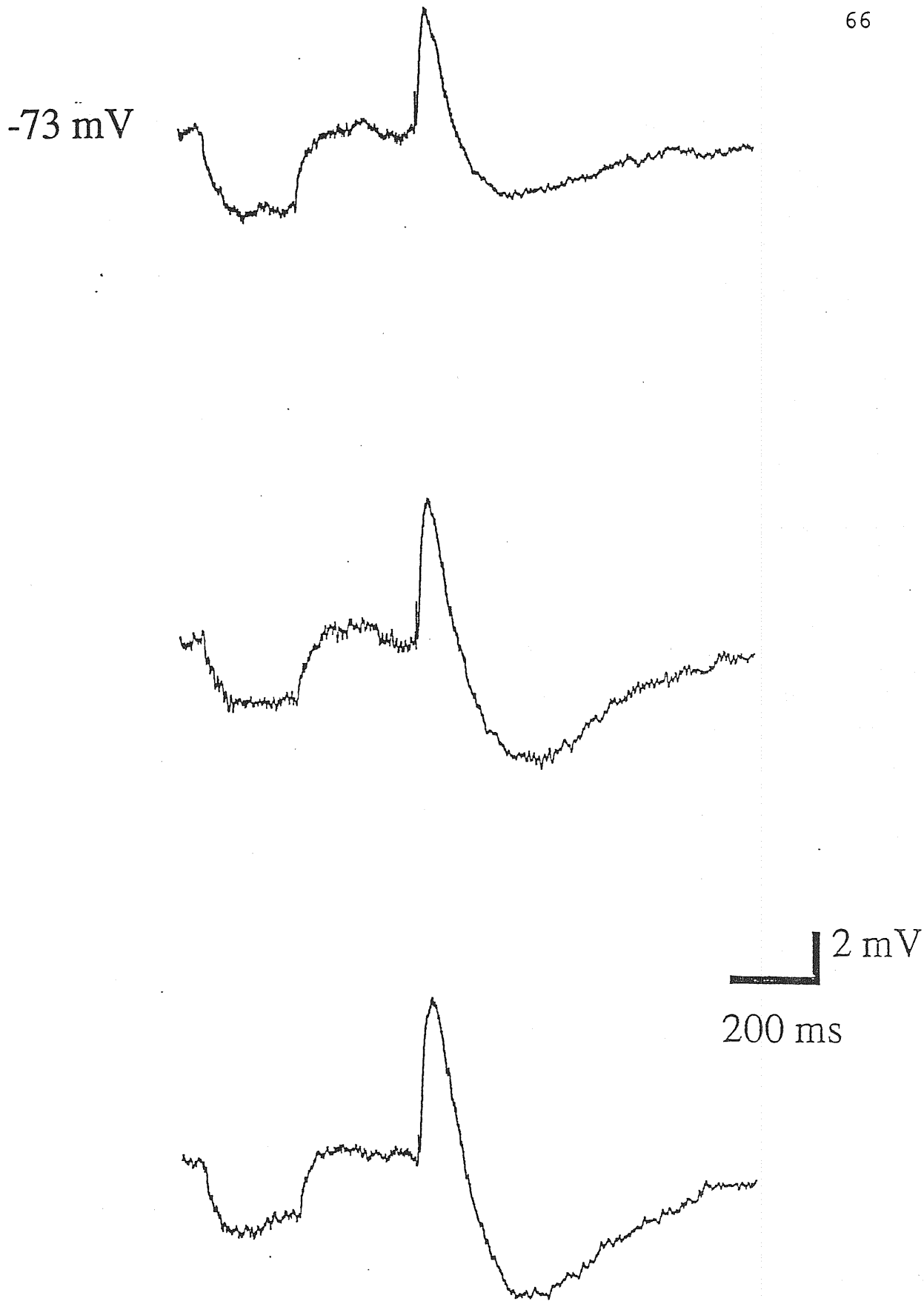


Fig. 22 Effect of high frequency stimulation on evoked EPSPs. Average of three single traces in control solution (10 µM picrotoxin). Upper trace recorded before (top), after 20 (middle) and 50 min (bottom) from delivery of the train. Hyperpolarizing pulse delivered to monitor Rm. High frequency stimulation was potentiating the area of the responses by 27 and 41% at 20 and 50 min respectively.

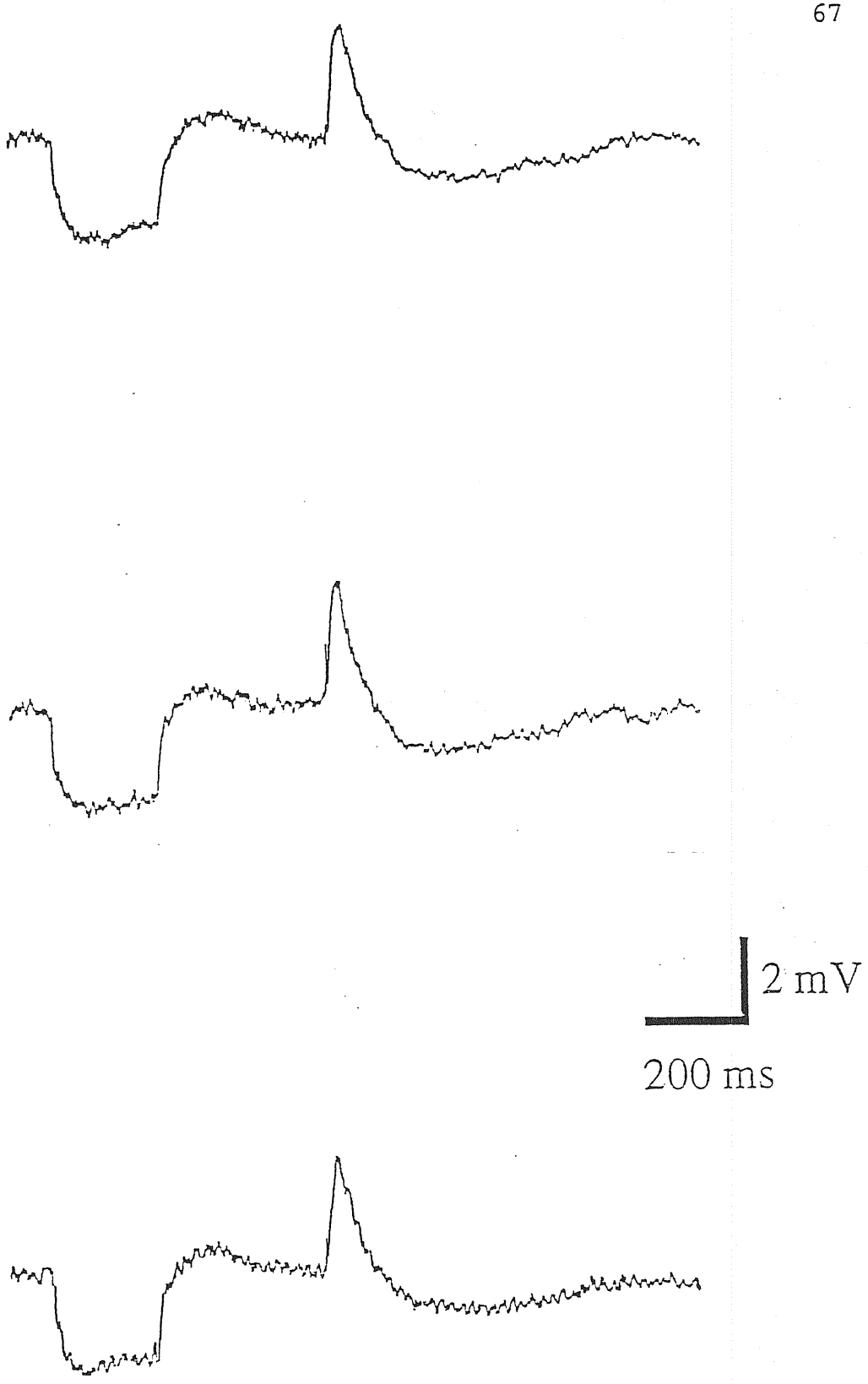


Fig. 23 Effect of TRH (10μM) on evoked EPSPs after high frequency stimulation. Average traces of three single traces in control (top), after 20 min (middle) and after 50 min (bottom) from the train. There is no significant change in the EPSP after high frequency stimulation.

rise time (15 ms and 17) remained unchanged both after 20 and 50 min being. After 50 min (top trace, Fig. 22) the responses were still potentiated and had a peak amplitude of 4.6 mV and an area of 270 mV*ms.

4.2 TRH MODULATION OF STP AND LTP

On 9 neurons TRH (10μM) was delivered 1 minute before the trains. After 20 min no significant changes were observed in the area or the rise time of the EPSPs, being 118±13% and 117±20% of pretrain control, respectively. Fig. 23 shows an example of the effect of the peptide: the lack of LTP development. The top trace of Fig. 23 represents one averaged EPSP recorded before the train, in control solution, out of a sample of 20 having peak amplitude, area and rise time of 3.1±0.0 mV,131±7 mV*ms and 8.5 ms respectively. After 20 min (central trace in Fig. 23) from TRH and trains delivery no potentiation was detected. The peak, area and rise time were 90% and 93% of control respectively. The same happened at 50 min after the trains, still no change was detected and the peak and the area were 72% and 74% of control. Note that in this neuron after tetanic stimulation the responses were on average depressed. Even in this set of experiment TRH was not affecting Rm.

DISCUSSION

1. GENERAL EFFECTS ON PYRAMIDAL NEURON INTRINSIC CONDUCTANCES

Unlike previous data which show a clear effect of TRH on neurons in other areas of the CNS, on hippocampal CA1 pyramidal neurons TRH modulated their activity in a more subtle way. No effects were observed at rest, when the cell was "quiescent". At more depolarized potentials, when the cell was activated for example by incoming excitatory drives, TRH started to exert its effects. We showed that the tripeptide depressed the AHP amplitude, which means that the conductance(s) activated during trains of spikes were decreased by TRH. The fact that both the fast and the slow conductances were affected (even if at different level) suggests that when one or more events reach firing threshold, the presence of TRH decreased the "refractory period"represented by the AHP, increasing the probability that a further event, single action potentials or trains of spikes, occurs. TRH could decrease the AHP via a secondary mechanism as for example a decrease in the HVA-I_{Ca2+} which underlay the activation of the Ca^{2+} dependent K^+ conductances. This interpretation seemed rather unlikely since no effect of TRH was observed on HVA-I_{Ca2+}.

Further information about the nature of this conductance was obtained after coapplication of TRH and 5HT. It has been shown that 5HT and TRH at high concentration abolished the AHP. In order to check whether the two drugs were sharing a common effector or if they were acting through completely independent effectors we applied these two drugs at concentrations at which they exert half-maximal effects. In these conditions the slow component of the AHP was almost abolished showing on average an occlusive effect between TRH and 5HT perhaps in view of a common effector mechanism.

2. POTENTIATION OF GLUTAMATERGIC DRIVE

The principal finding of the present study was the large, albeit transient, potentiation by TRH of synaptic responses mediated by glutamate via NMDA receptors. There seems to be little doubt regarding the specificity of this phenomenon for the NMDA receptors as the other major receptor classes involved in synaptic transmission were pharmacologically blocked by a combination of selective antagonists (CNQX, bicuculline and CGP 35348). This effect of TRH was temporary as the size of the EPSP returned to baseline level despite the continuous application of the neuropeptide. Since AMPA/kainate receptor-mediated potentials (in the presence of NMDA receptor block) were found to be insensitive to TRH, this observation strenghtens the specificity of this neuropeptide towards NMDA receptor-mediated excitatory synaptic transmission. This action of TRH was investigated with intracellular recording under current clamp conditions in order to minimize space clamp problems inherent in voltage clamping widely distributed synaptic inputs (Major, 1993; Spruston et al., 1994) and to reduce the risk of rapid rundown of TRH-induced responses under whole cell patch clamp conditions (Dufy et al., 1986). Since NMDA receptors activity might be regulated by altering the voltage-sensitivity of the NMDA receptor channels, it was deemed important to examine the action of TRH on cells bathed in a solution containing Mg^{2+} and at their normal resting potential. The results obtained with the evoked potentials are thus in keeping with the transient potentiation of NMDA-induced depolarizations of hippocampal CA1 neurons. Moreover in the latter case a modulation of the Mg^{2+} block was suggested.

The present data raise two questions: a, the *locus* of action of TRH in potentiating NMDA receptor mediated responses; b, the mechanism(s) reponsible for this phenomenon. As far as the first question is concerned, it seems likely that TRH was facilitating NMDA receptor mediated EPSPs at postsynaptic level rather than promoting release of endogenous glutamate. This

interpretation is based on the fact that no corresponding change in AMPA/kainate receptor mediated EPSPs was observed, which argues against any large presynaptic increase in glutamate release which should have affected both NMDA and nonNMDA receptors in view of their proposed colocalization (McBain and Dingledine, 1992). Furthermore, in baseline conditions synaptically-released glutamate is thought to be sufficient to saturate NMDA receptors (Kullmann, 1994), a phenomenon which would not favour modulatory effects by TRH simply increasing glutamate release. Nevertheless, in the present study the presynaptic hypothesis cannot be entirely dismissed because more rigorous tests, based on quantal analysis of synaptic transmission, were not possible due to the very low rate of electrical stimulation and the rare occurence of spontaneous synaptic events. Some presynaptic contribution to the action of TRH remains thus possible but it is perhaps more relevant when responses to exogenous NMDA rather than synaptic potentials are investigated as the former are partially sensitive to tetrodotoxin block. The question of the mechanism of action of TRH has not been directly addressed in the present study and can only be surmised from data obtained from this and other investigations. It seems unlikely that the potentiating action of TRH merely resulted from an increased resistance of the postsynaptic membrane or from membrane depolarization (sufficient to attenuate the Mg^{2+} block of NMDA channels; Ascher and Nowak, 1988) as no change in either of these parameters was detected in the present or former experiments (Ballerini et al, 1994). It appears thus feasible that TRH upregulated NMDA-gated channel activity rather than the receptor number, as the effect developed rapidly. Since TRH binds to its own set of distinct membrane receptors (Sharif, 1989), this upregulation was probably caused via an intracellular signal transduction pathway, for example by stimulation of hydrolysis of inositol phospholipids which has been observed in pituitary cells (Gershengorn, 1986) and hippocampal neurones (Ebihara and Akaike, 1994). These effects may lead to NMDA receptor phosphorylation and consequent amplification of synaptic

responses (Raymond et al., 1993). Exhaustion of intracellular metabolites and/or TRH receptor tachyphylaxis (Fisher and Nistri, 1993) might account for the transient nature of the TRH-induced potentiation, which can be regarded as a chemically-evoked form of short term change in efficacy of NMDA receptor-dependent synaptic transmission (Malenka and Nicoll, 1993).

Physiological Significance of potentiation by TRH

The action of TRH towards NMDA receptor mediated responses is probably not restricted to the CA1 region of the hippocampus. In fact, it has also been found in the case of exogenously-applied NMDA to brainstem neurones (Rekling, 1992) or of NMDA receptor-mediated synaptic transmission of extracellularly-recorded spinal neurones (Chizh and Headly, 1994). The presence of this phenomenon outside hippocampal tissue suggest that it may be a form of NMDA receptor regulation by this endogenous peptide fairly widespread in the central nervous system. Moreover the modulation by TRH of a highthreshold Ca^{2+} dependent K^+ current of hippocampal cells detectable at membrane potentials positive to about -40 mV (Ballerini et al., 1994) could contribute to a further upregulation of NMDA receptor mediated responses due to an increase in the excitability of pyramidal cells over a wide range of membrane potentials without changing directly the passive properties of the neuronal membrane.

3. REDUCTION OF GABAERGIC DRIVE

TRH-induced decrease in synaptic inhibition

The principal finding of this study is that TRH downmodulated evoked GABAergic fast and slow IPSPs of CA1 neurones of the rat hippocampal slice preparation. It is now well established that fast IPSPs of hippocampal pyramidal neurones underlying feed-forward or feed-back inhbition are mediated by GABA acting on $GABA_A$ receptors (Sivilotti and Nistri, 1991). Single inhibitory fibres are thought to release GABA which then binds to $GABA_A$ and $GABA_B$ receptors of

pyramidal cells (Alger, 1991). In the present study $GABA_A$ receptor mediated fast IPSPs were evoked by stimulation of GABAergic cells in the LM layer or in the pyramidal layer, and were pharmacologically isolated after blocking glutamatergic EPSPs. It was difficult to observe $GABA_B$ receptor mediated IPSPs in isolation particularly when stimuli were applied to the LM layer. In many tests on slow IPSPs it was therefore necessary to preserve the fast, AMPA receptor mediated EPSP which, as shown in previous studies (Kasparov et al., 1994), was fully insensitive to TRH.

Characteristics of TRH action

Several aspects of the depressant action of TRH on IPSPs are noteworthy. First, when tested at the same concentration producing many fold increases in slow NMDA mediated EPSPs, TRH decreased but never abolished either fast or slow IPSPs. The attenuation of GABAergic transmission developed in the absence of changes in passive membrane properties or IPSP reversal potential, thus excluding a non-specific action by the peptide. Furthermore, it is unlikely that this observation was due to modulation by TRH of $GABA_A$ receptors either directly or indirectly by raising for instance intracellular Ca^{2+} (Ebihara and Akaike, 1993) and thus promoting receptor desensitization (Stelzer et al., 1988). In fact, responses to the selective $GABA_A$ agonist isoguvacine (either in control or TTX solution) were unaffected by TRH. Similar data were also observed in the case of baclofen, confirming that TRH did not affect postsynaptic $GABA_B$ receptors directly. The reduction in GABAergic transmission seems to be best explained by a presynaptic site of action of the peptide on the mechanism(s) of GABA release from afferent fibres to pyramidal neurones. This suggestion would be strengthened by observing a decrease in the frequency of spontaneous IPSPs but their small amplitude and low occurrence made it difficult to obtain such a validation since data sampling was biased towards the largest events. Future studies using other methods with improved signal/noise ratio (such as whole cell patch clamping)

might prove useful to corroborate these findings. While sharp microelectrode recordings were therefore impractical for studying spontaneous synaptic events, they provided the advantage of minimal disturbance to the intracellular milieu of the neurones and thus ensured long-term reliable recordings of evoked GABA-mediated responses with no run-down. Certainly, there was no evidence that TRH reduced fast IPSPs by direct interference with presynaptic $GABA_B$ receptors controlling GABA release since the IPSP depression was observed during pharmacological block of $GABA_B$ receptors. There is also no evidence for an effect of TRH on highthreshold K^+ currents of GABAergic interneurones like the one found on pyramidal cells (Ballerini et al., 1994): had this phenomenon taken place, it should have increased excitability and hence GABA release, a result contrary to the observed IPSP depression. The TRH sensitivity of fast IPSPs originating by stimulating two different areas would also exclude a specific effect of the peptide restricted to a subset of GABAergic cells. While the present data allow to rule out some possible mechanisms responsible for the effect of TRH, the intimate processes by which TRH impaired synaptic inhibition remain uncertain.

Differential sensitivity of fast and slow IPSPs to TRH

TRH affected only slow IPSPs originating from LM and not those arising from SP. The regional variation in slow IPSP sensitivity cannot be simply due to the dissimilar distribution of TRH receptors as fast IPSPs elicited from the same area were not less sensitive to TRH. It seems more likely that the differential sensitivity of slow IPSPs to TRH reflects some intrinsic characteristics of these responses rather than any particular topography of action by the peptide. Perhaps the amount of synaptically released GABA was saturating the number of $GABA_B$ receptors exposed to this transmitter. Only quite considerable reductions in GABA release would then induce a strong depression of slow IPSP. This phenomenon might be equivalent to what in classical pharmacological terms is called "receptor reserve", namely that only a small fraction of available

receptors needs to be occupied to elicit a full tissue response. Hence, spare $GABA_B$ receptors may ensure apparent insensitivity of SP slow IPSPs and less sensitivity of LM slow IPSPs than their concurrent fast IPSPs.

4. REDUCTION BY TRH OF STP AND LTP

Even if the body of data presented is still preliminary an interesting effect of TRH on the short and long term modification have been observed. The fact that TRH was decreasing K^+ conductances, transiently upregulating NMDA mediated responses and downregulating the GABAergic drive was suggesting an overall strong tendency to enhance excitatory drives to pyramidal neurons. On the other hand in our experiments TRH limited the development of LTP. Even if further investigation are still required, some hypothesis can be infered to explain such effect.

During the induction of STP/LTP a large amount of glutamate is released and a large number of NMDA receptors are activated alltogether causing a massive increase in $[Ca^{2+}]_i$. Moreover TRH is very likely to increase by itself the basal level of intracellular Ca^{2+} during the time of its application. When the train is delivered to the cell the level of intracellular Ca^{2+} can be already high. The lack of development of further potentiation induced by tetanic stimulation, could suggest that in the case of a previous and transient upregulation of NMDA receptor was already induced by TRH application, no further effect could be induced by the following stimulations. In other words TRH might cause the cell to enter a sort of "refractory" mode. This would be in agreement with some clinical and experimental studies suggesting a protective function of TRH in cases of strong activation like epileptic phenomena (Sato et al., 1984; Ueda et al., 1983).

More difficult interpretation may be done of GABAergic modulation since it does still seem controversial. As far as the evoked GABAergic drive is concerned its downregulation would contribute to the overall enhancement previously suggested.

The increase of the spontaneous GABAergic drive (observed under some experimental condition) having a slower onset could intervene in a more delayed phase when the potentiation of NMDA receptor is over contributing to the further inhibition.

5. SIGNIFICANCE OF TRH-INDUCED EFFECTS

Relevance of TRH action to microphysiology of CA1 neurones

A strong, albeit transient, upregulation of NMDA receptors by TRH may represent an important process to increase the excitability of CA1 neurones. This phenomenon would be reinforced by the concomitant reduction in fast GABAergic inhibition, making the cell membrane more responsive to fast excitatory inputs while slow signals could still be attenuated by relatively unaffected slow IPSPs. Moreover it would amplify small excitatory inputs to be closer to spike threshold. Spike bursting would then be encouraged by the depression of the slow afterhyperpolarization. In this way the firing of CA1 neurones would be tuned up while intrinsic inhibitory conductances and residual synaptic inhibition would still prevent run-away excitation.

While in the case of a particularly strong enhancement of activity, as it happens during tetanic stimulations, TRH potentiatory effects on CA1 neurons may lead to an overexcitation followed by an "inactivation"and a final damping action.

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