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Factors affecting the time course of excitatory and inhibitory synaptic transmission in the hippocampus

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Factors Affecting the Time Course of Excitatory and Inhibitory Synaptic Transmission in the Hippocampus

by

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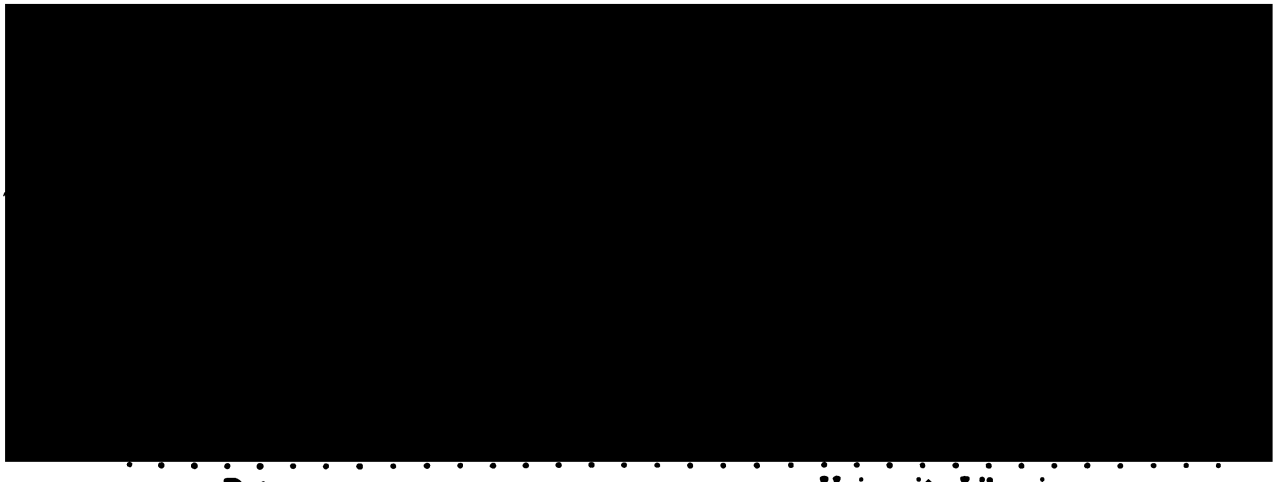

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Factors affecting the time course of excitatory and inhibitory synaptic transmission in the hippocampus

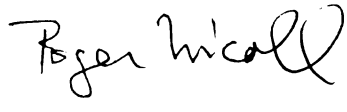
Jeffry Seth Isaacson

The factors governing the time course of synaptic transmission remain a fundamental question in neurophysiology. This issue is of great importance since the strength of neurotransmission is dependent on the duration of the synaptic response. While much is known about the factors underlying the duration of the synaptic response at the neuromuscular junction (NMJ), the factors involved in the central nervous system are still unclear. In the brain, a number of mechanisms, including the kinetics of the postsynaptic receptors and transmitter clearance by diffusion or via specific uptake systems, have been proposed to govern the time course of synaptic transmission. Furthermore, little is known about the behavior of the transmitters themselves, in particular, their effective lifetimes and the spatial domains over which they can act. In this study, we have used patch clamp techniques in brain slices to address some of these issues at both excitatory and inhibitory synapses in the hippocampus.

We have found that aniracetam, a 2-pyrol-lidinone compound, selectively prolongs the time course of fast excitatory synaptic transmission. In outside-out patches, aniracetam effects both the factors that govern the channel closing rate of glutamate receptors and the entry of the receptors into a desensitized state. These results are in agreement with proposals used to account for the time course of other fast synapses, such as the NMJ, that a

transient rise in transmitter concentration generates a synaptic current whose decay is governed by the intrinsic kinetics of the postsynaptic receptors.

We have also examined the time course of inhibitory synaptic transmission in the hippocampus. We have found that transmitter uptake limits the strength of postsynaptic inhibition, in part by regulating the spill-over of transmitter from adjacent synapses. This result suggests that the effective spatial domain of transmitter action in the brain can extend beyond the borders of the synaptic cleft. Furthermore, we have found that synaptically-released GABA can act in a diffuse, paracrine manner to inhibit excitatory transmission through a presynaptic mechanism. As is the case for postsynaptic inhibition, this presynaptic "action at a distance" in the hippocampus is also strongly influenced by transmitter uptake.



Dr. Roger A. Nicoll, M.D.
Committee Chairman

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General Introduction

I. Synaptic Transmission in the Hippocampus

Anatomy

Our understanding of synaptic transmission in the central nervous system (CNS) has advanced considerably following the development of techniques permitting the study of brain slices *in vitro* (Dingledine, 1984). One of the best studied preparations, the hippocampal slice, provides the ability to study monosynaptic excitatory inputs from anatomically distinct pathways onto a number of different cell types: the perforant path inputs to granule cells in the dentate gyrus, mossy fiber synapses onto CA3 pyramidal cells, and Schaffer collateral/commissural inputs onto CA1 pyramidal cells (Fig. 1A). In addidtion to these excitatory synapses, inhibitory input to these principle cells is provided by local interneurons. In area CA1, an overly simplified schematic of the synaptic inputs to a pyramidal cell (Fig. 1B) illustrates that, in addition to the *en passant* excitatory synapses on the dendrites of the cell, two classes of interneurons have been proposed to generate synaptic inhibition (Alger & Nicoll, 1982). One class of interneurons, which contacts the soma and initial segment of pyramidal cells (Somogyi, Smith, Nunqi, Gorio, Takagi & Wu, 1983), is thought to mediate feedback inhibition, while a separate class of feedforward interneurons (Alger & Nicoll, 1982; Lacaille & Schwartzkroin, 1988a; Lacaille & Schwartzkroin, 1988b; Williams & Lacaille, 1992) are thought to generate dendritic inhibitory responses onto these cells.

Excitatory Synaptic Transmission

In the hippocampus and much of the CNS, the amino acid L-glutamate is commonly considered to be the neurotransmitter mediating fast excitatory synaptic transmission. Many findings support the role of glutamate: 1) glutamate strongly excites neurons, and antagonists selective for glutamate receptors block both the actions of exogenous glutamate and excitatory transmission; 2) nerve terminals contain glutamate, apparently in synaptic vesicles; and 3) the Ca^{2+}-dependent release of glutamate has been detected in a number of experimental models (Collingridge & Lester, 1989; Cotman & Nadler, 1981; Dunlop, Fear & Griffiths, 1989; Mayer & Westbrook, 1987b; Nicoll, Malenka & Kauer, 1990).

The glutamate receptors underlying conventional excitatory transmission are separated into two classes of ligand-gated ion channels based on their preference for different agonists: the receptors responding to N-methyl-D-aspartate (NMDA receptors) and those that prefer the agonists amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), quisqualate, and kainate (non-NMDA receptors) (Collingridge & Lester, 1989; Mayer & Westbrook, 1987b). Recent molecular biological studies have identified families of genes encoding ligand-gated channels corresponding to the two subtypes of glutamate receptors (Boulter, Hollmann, O'Shea-Greenfield, Hartley, Deneris, Maron & Heinemann, 1990; Egebjerg, Bettler, Hermans-Borgmeyer & Heinemann, 1991; Hollmann, O'Shea-Greenfield, Rogers & Heinemann, 1989; Keinånen, Wisden, Sommer, Werner, Herb, Verdoorn, Sakmann & Seeburg, 1990; Moriyoshi, Masu, Ishii, Shigemoto, Mizuno & Nakanishi, 1991; Nakanishi, Shneider & Axel, 1990).

While both types of receptor can be colocalized at the same synaptic site (Bekkers & Stevens, 1989; Robinson, Sahara & Kawai, 1991; Silver, Traynelis & Cull-Candy, 1992) and therefore have access to the same synaptically-released transmitter, they have very different properties and have distinctly different contributions to excitatory transmission.

NMDA receptors have received considerable attention for their important role in the induction of such diverse phenomena as synaptic plasticity (Collingridge & Singer, 1990; Madison, Malenka & Nicoll, 1991) and neurotoxicity (Choi, 1988; Rothman & Olney, 1987). Studies of this receptor type have revealed a number of interesting properties including: a dependence on glycine binding for channel activation (Johnson & Ascher, 1987); a voltage-dependent block of the channel by Mg^{2+} (Mayer, Westbrook & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984); and a high permeability of the channel to Ca^{2+} (MacDermott, Mayer, Westbrook, Smith & Barker, 1986; Mayer, MacDermott, Westbrook, Smith & Barker, 1987; Mayer & Westbrook, 1987a). The voltage-dependent block by Mg^{2+} prevents the activation of the NMDA receptor at hyperpolarizing membrane potentials. Thus, under normal conditions in the hippocampus when neurons have resting membrane potentials of -55 to -70 mV the contribution of NMDA receptors to low frequency synaptic transmission is presumably very small. In support of this hypothesis, normal, low-frequency excitatory transmission is nearly entirely blocked by the non-NMDA receptor specific antagonists 6-cyano-7-nitroquinoxaline-2.3-dione (CNQX) or 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Collingridge & Lester, 1989).

Non-NMDA receptors mediate normal fast synaptic transmission at many synaptic pathways in the CNS (Collingridge & Lester, 1989). A number of studies suggest that the modulation of these receptors underlies

the changes in synaptic efficacy associated with different models of synaptic plasticity in the hippocampus and cerebellum (Davies, Lester, Reymann & Collingridge, 1989; Linden, Dickinson, Smeyne & Connor, 1991; Manabe, Renner & Nicoll, 1992). Most of the non-NMDA receptors characterized in the CNS form ion channels that are mainly permeable to monovalent cations (Collingridge & Lester, 1989; Mayer & Westbrook, 1987b). Perhaps their most interesting property is that the response of these receptors rapidly diminishes in the continued presence of agonists (Trussell, Thio, Zorumski & Fischbach, 1988). Rapid perfusion experiments using cultured neurons have found that this desensitization of the receptors to agonist occurs extremely rapidly, beginning a few milliseconds after glutamate is applied (Kiskin, Krishtal & Tsyndrenko, 1986; Mayer, 1989; Mayer & Vyklicky, 1989; Tang, Dichter & Morad, 1989; Trussell & Fischbach, 1989).

Inhibitory Synaptic Transmisison

In the brain, GABA is widely recognized as the most ubiquitous neurotransmitter of inhibitory synaptic transmission (Nicoll et al., 1990). Two types of GABA receptors, $GABA_A$ and $GABA_B$, have been characterized and both mediate postsynaptic inhibition in the hippocampus. $GABA_A$ responses are mimicked by the agonists muscimol and 4,5,6,7-tetrahydroisoxazolo(5,4-c) pyridine-3-ol (THIP) and blocked by the competitive antagonist bicuculline (Curtis, Duggan, Felix & Johnston, 1971), as well as by picrotoxin which is a non-competitive inhibitor (Twyman, Rogers & Macdonald, 1989). Baclofen is the best described $GABA_B$ receptor agonist (Bowery, 1989) and CGP 35348 is currently the most effective competitive inhibitor of this class of receptors (Olpe, Karlsson, Pozza, Brugger, Steinmann, Van Riezen, Fagg, Hall, Froestl & Bittiger, 1990).

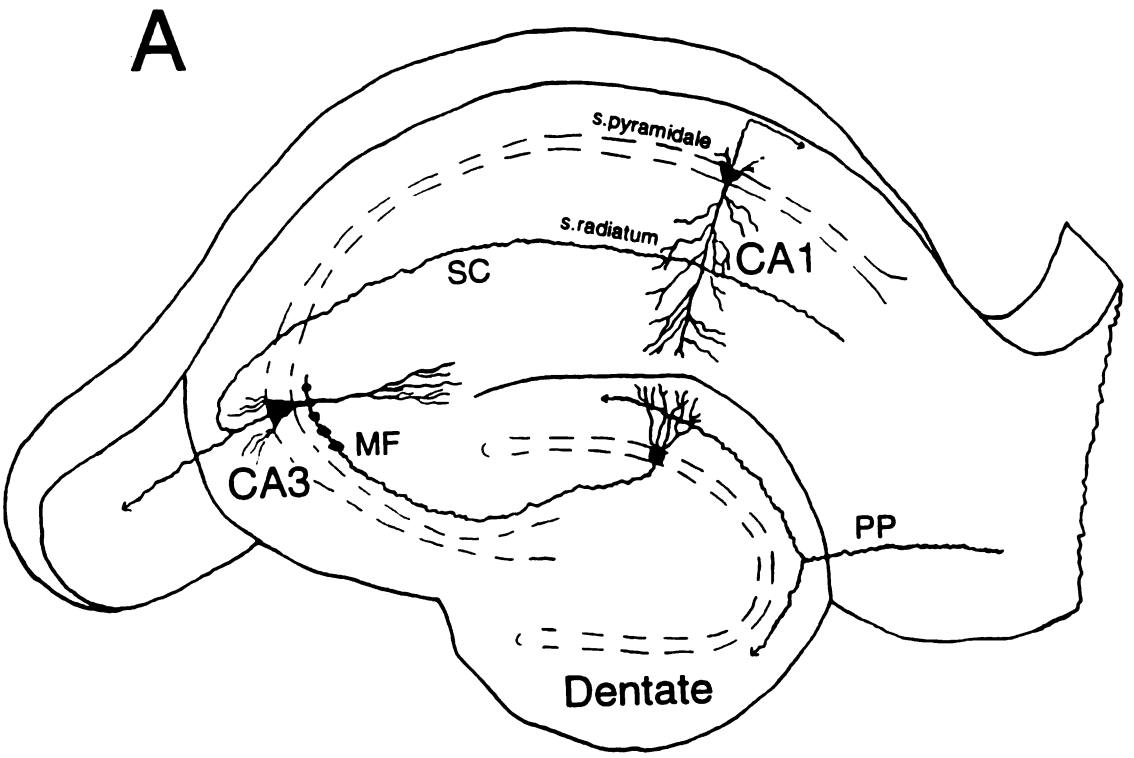
In general, the properties of the GABA receptors underlying synaptic inhibition in the hippocampus are well known. The $GABA_A$ receptor was one of the first neurotransmitter receptors whose cDNA was cloned and sequenced (Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg & Barnard, 1987). However, the functional role of these receptors was known long before the genes encoding $GABA_A$ receptors were identified. $GABA_A$ receptors are well established as coventional ligand-gated ion channels that permit Cl^- ion entry into neurons, hyperpolarizing the cell and making it less likely to reach threshold for firing an action potential (Nicoll et al., 1990). In contrast, $GABA_B$ receptors are coupled to GTP binding proteins and hyperpolarize neurons by activating a K^+ conductance (Andrade, Malenka & Nicoll, 1986; Gahwiler & Brown, 1985; Newberry & Nicoll, 1984a; Newberry & Nicoll, 1984b; Newberry & Nicoll, 1985). In addition to its postsynaptic action, baclofen, acting through $GABA_B$ receptors, also causes a presynaptic inhibition of both excitatory and inhibitory transmission (Ault & Nadler, 1982; Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull, 1980; Dutar & Nicoll, 1988b; Harrison, 1990; Inoue, Matsuo & Ogata, 1985; Lanthorn & Cotman, 1981; Olpe, Baudry, Fagni & Lynch, 1982).

Unlike the case with glutamate receptors, there is good evidence for the segregation of $GABA_A$ and $GABA_B$ receptors at different postsynaptic locations (Otis & Mody, 1992a; Sugita, Johnson & North, 1992). In the hippocampus, $GABA_B$ responses are most often localized to the dendrites of pyramidal and granule cells, while $GABA_A$ responses predominate at the somata of these cells (Newberry & Nicoll, 1985). In agreement with these results, synaptically-released GABA has been shown to evoke predominantly $GABA_A$-mediated inhibition at the soma (Alger & Nicoll, 1982; Lacaille,

Mueller, Kunkel & Schwartzkroin, 1987; Solís & Nicoll, 1992b) and $GABA_B$-meditated responses in the dendrites (Lacaille & Schwartzkroin, 1988b; Segal, 1990; Solís & Nicoll, 1992b; Williams & Lacaille, 1992). These results support the proposal (Alger & Nicoll, 1982) that feedback interneurons generate a predominantly $GABA_A$ mediated fast inhibition at the soma while feedforward interneurons generate $GABA_B$ mediated slow dendritic inhibition.

Figures

Fig. 1 Schematic diagram of synaptic pathways in a transverse hippocampal slice. *A*, synaptic pathways and principle target cells in the transverse slice. PP, perforant path; MF, mossy fiber; SC, Schaffer collateral. (Adapted from Madison, 1983). *B*, simplified schematic of the synapses formed onto a pyramidal cell from *en passant* excitatory afferent fibers and inhibitory interneurons. (After Alger & Nicoll, 1982).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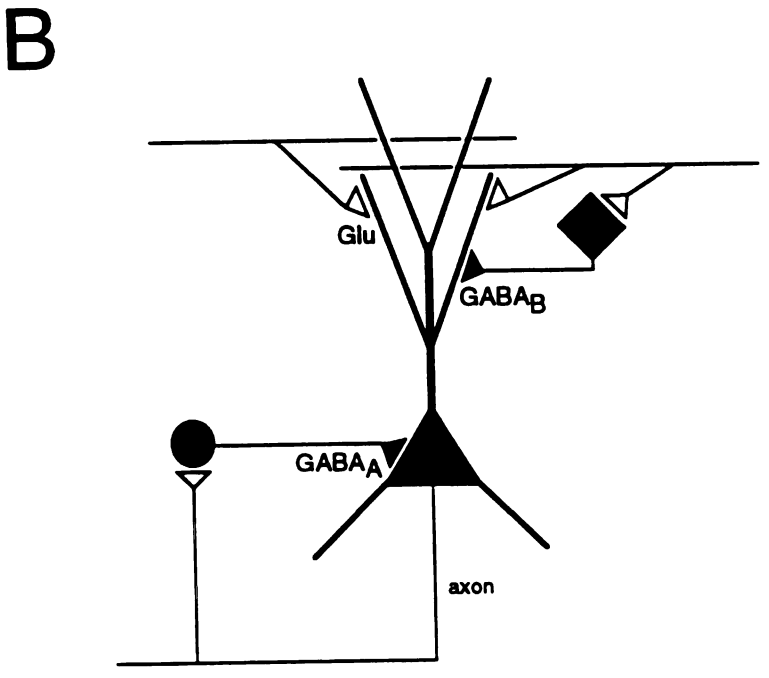
B
Glu
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GABA$_A$
axon

II. The Time Course of Synaptic Transmission

The modulation of the time course of chemical transmission represents a powerful mechanism for controlling synaptic strength. It is not suprising then, that a fundamental question in neurophysiology concerns the factors governing the duration of the synaptic response.

The Neuromuscular Junction

Pioneering studies of the neuromuscular junction (NMJ) provide the basis of our understanding of the control of the time course of synaptic transmission (Martin, 1977; Takeuchi, 1977). At the NMJ, stimulation of the nerve leads to the release of synaptic vesicles from a number of release sites. The transmitter, acetylcholine (ACh), crosses the synaptic cleft by diffusion and activates postsynaptic receptors that generate a fast synaptic current. Eccles and Jaeger calculated the diffusion of transmitter in synaptic models and concluded that it is rapid enough to account for the uniform distribution of ACh within the synaptic cleft in a few microseconds (Eccles & Jaeger, 1958). Under normal conditions, the rising phase of the synaptic current at the NMJ is complete within 1 - 2 ms and the decay of the synaptic current can be described by a single exponential function with a tau value of a few ms. The rapid rise of the synaptic response presumably reflects the kinetics of transmitter binding and opening of the ligand-gated receptors. However, a number of factors at the NMJ have been considered to influence the decay of the synaptic current: 1) transmitter removal by enzymatic destruction, 2) transmitter removal by diffusion, and 3) the dissociation of the transmitter-receptor complex and the intrinsic kinetics of ACh channel closure.

If the rate-limiting step in the decay of the end-plate current was due to simple diffusion, then the time course of the current should not be strongly affected by temperature. Magelby & Stevens tested this hypothesis by examining the decay of the end-plate current over a range of temperatures (Magleby & Stevens, 1972b). They found that the decay constant of the synaptic current had a Q_{10} of 2.7, indicating that a process more complicated than the diffusion of transmitter out of the cleft accounts for the time course of the synaptic response.

An interesting feature of the NMJ is that acetylcholine is rapidly hydrolyzed by the degradative enzyme acetylcholinesterase (Kandel, Schwartz & Jessell, 1991; Scheller & Hall, 1992). It has been well established that inhibitors of this enzyme greatly prolong the time course of the synaptic current (Eccles, Katz & Kuffler, 1942; Eccles & McFarlane, 1949; Katz & Miledi, 1973; Katz & Thesleff, 1957; Magleby & Stevens, 1972a). In the presence of cholinesterase inhibitors, miniature end-plate currents, representing the response to individual vesicles of transmitter, can last for tens of milliseconds, a time that is longer than the lifetime of transmitter in the synaptic cleft predicted by the diffusion equation (Eccles & Jaeger, 1958). This discrepancy was addresed in a study by Katz & Miledi that indicated that, in the absence of cholinesterase, ACh molecules can bind to receptors several times before diffusing out of the synaptic cleft (Katz & Miledi, 1973). It was suggested that the repeated binding of Ach to its receptors would effectively slow the disappearance of transmitter from the synaptic cleft. However, this "buffered diffusion" does not occur when cholinesterase is intact, since after its initial binding ACh is quickly degraded before it can interact with another receptor.

If the actions of acetylcholinesterase and diffusion are both very fast relative to the time course of the synaptic current, can another mechanism account for the decay of the synaptic current under normal conditions? One possibility is that the intinsic kinetics of the ACh receptor, such as the channel closing rate and dissociation rate of transmitter, determine the time course of the synaptic response. Strong support for this hypothesis was found by studying the current fluctuations produced by ACh at the neuromuscular junction (Anderson & Stevens, 1973). Anderson & Stevens used analyses of the power spectra of "ACh noise" to yield the amplitude and duration of the channel events underlying the fluctuations. The decay of the ACh-induced current fluctuations could be described by a single exponential function with a time constant identical to the one used to fit the decay of the nerve-evoked synaptic current. More recently, single channel studies (Sakmann, Patlak, & Neher, 1980; Colquhoun & Sakmann, 1985), have confirmed the similarity between channel lifetime and the decay time constant of the endplate potential.

Further evidence in support of a role for channel kinetics was provided by a number of groups that found that the decay of the synaptic current at the NMJ (Kordas, 1969; Magleby & Stevens, 1972a; Takeuchi & Takeuchi, 1959), as well as the response to exogenously-applied transmitter (Anderson & Stevens, 1973; Dionne & Stevens, 1975) was voltage-dependent. The simplest interpretation of this result is that the lifetime of the open channel is influenced by the surrounding membrane potential. Thus, under normal conditions, when ACh interacts briefly with postsynaptic receptors before its removal from the synaptic cleft, properties of the receptor-channel that govern the open channel lifetime such as the channel closing rate and

transmitter dissociation rate, can account for the rapid decay of the synaptic current at the NMJ.

Whereas the NMJ represents a model system for studying the time course of synaptic transmission, a number of features make it quite distinct from synapses in the CNS. First, as opposed to the multiple release sites and large number of postsynaptic receptors at the NMJ, it is believed that in the CNS synaptic boutons contain one or a small number of release sites that access small clusters of postsynaptic receptors (Edwards, 1991; Korn & Faber, 1991; Redman, 1990). Second, transmitters such as glutamate and GABA are not enzymatically degraded in the extracellular space. Rather, both of these transmitters are cleared from the extracellular environment by high affinity uptake systems in neurons and glia (Hertz, 1979; Nicholls & Attwell, 1990). Thus, in the CNS where the enzymatic destruction of transmitter does not occur, transmitter diffusion or its reuptake into surrounding cells might have an important influence upon the time course of synaptic transmission.

CNS: Excitatory Synaptic Transmission

A number of studies using patch-clamp techniques in brain slices have investigated the kinetics of NMDA receptor- and non-NMDA receptor-mediated synaptic currents. The time course of the two currents are quite distinct, the synaptic current through non-NMDA receptors lasts a few milliseconds, whereas NMDA receptor-mediated responses peak in about 20 ms and can last for hundreds of ms (Hestrin, Nicoll, Perkel & Sah, 1990; Hestrin, Sah & Nicoll, 1990; Randall, Schofield & Collingridge, 1990; Stern, Edwards & Sakmann, 1992).

The mechanism generating the slow rise and decay of NMDA receptor-mediated excitatory synaptic currents (EPSCs) has recently been established. Hestrin et al. showed in the hippocampal slice that open channel blockers,

known to modify NMDA channel kinetics, reduced the EPSC decay time (Hestrin et al., 1990). This result and others in the study led them to support a model in which the rise and decay time of the NMDA component of the EPSC are determined primarily by the slow kinetics of NMDA channels. Direct evidence in support of this hypothesis was shown by Lester et al. (Lester, Clements, Westbrook & Jahr, 1990) using the rapid application of glutamate to outside out patches from cultured hippocampal cells. They found that brief applications of glutamate to NMDA receptors generated responses that mimicked the slow rise and decay of NMDA receptor-mediated synaptic currents. Thus, the intrinsic kinetics of the opening and closing of the NMDA receptors could account for the slow time course of NMDA receptor-mediated synaptic responses.

At first, the time course of the non-NMDA receptor-mediated EPSC appears less interesting due to its similarity to the end-plate current of the NMJ. Like the NMJ, the decay of the non-NMDA EPSC has a high Q_{10}, suggesting that it is not determined by free diffusion of transmitter (Hestrin et al., 1990). Since a very brief rise in transmitter concentration at the same synapse can account for the NMDA component of the EPSC, it is easy to imagine that the decay of the non-NMDA receptor current could simply reflect the rapid dissociation of transmitter from these receptors. However, the decay of the synaptic current mediated by non-NMDA receptors is not voltage-sensitive (Hestrin et al., 1990) so that direct evidence for the role of channel kinetics has not been firmly established. Furthermore, rapid perfusion experiments with non-NMDA receptors indicate that this receptor desensitizes extremely quickly in the presence of glutamate (Kiskin et al., 1986; Mayer & Vyklicky, 1989; Patneau & Mayer, 1991; Tang et al., 1989; Trussell & Fischbach, 1989) with a time course similar to that of glutamate-

mediated synaptic responses. This similarity has led to the proposal that the rapid desensitization of the receptor contributes to the decay of the synaptic current (Tang et al., 1989; Trussell & Fischbach, 1989). The precise role of channel kinetics in determining the time course of non-NMDA receptor-mediated synaptic transmission has yet to be firmly established.

CNS: Inhibitory Synaptic Transmission

In hippocampal neurons, fast inhibitory postsynaptic currents (IPSCs) mediated by $GABA_A$ receptors have a fast rise time of about 1 ms and decay exponentially with a tau of about 20 ms (Collingridge, Gage & Robertson, 1984; Otis & Mody, 1992b; Segal & Barker, 1984). In some cases, the IPSCs show a double-exponential decay with the initial decay of the synaptic current fitted by an additional exponential function with a tau of 1-3 ms (Edwards, Konnerth & Sakmann, 1990). Utilizing the criteria mentioned previously, a number of results indicate that channel kinetics determine the time course of fast inhibitory transmission: 1) the decay of the IPSC is voltage-sensitive (Collingridge et al., 1984), 2) the IPSC has a high Q_{10} (Collingridge et al., 1984), and 3) pharmacological agents, such as barbiturates and benzodiazepines, which increase $GABA_A$ channel open time or burst duration prolong the decay of IPSCs (Otis & Mody, 1992b; Segal & Barker, 1984)

The factors governing the slow rise and decay of $GABA_B$ receptor mediated synaptic currents are much less clear. However, the time course of the slow IPSC in the hippocampus is remarkably similar to other slow synaptic potentials mediated by a_2-adrenergic, M_2 muscarinic, $5\text{-}HT_{1A}$ serotonin, D_2 dopamine, and somatostatin receptors (Hille, 1992; Nicoll, 1988; North, 1989). In each of these cases activation of these receptors causes the opening of a K^+ channel that is triggered by a direct interaction with pertussis toxin-sensitive G proteins (Hille, 1992). It is generally believed that the slow

time course of these synaptic potentials is determined by the molecular interactions involved in the receptor-G-protein-channel coupling (Hille, 1992; Nicoll et al., 1990). More specifically, the decay of the slow IPSC could reflect slow dissociation of agonist from its receptor or the time course of hydrolysis of activated G-protein subunits.

Transmitter Uptake

The removal of Ach from the synaptic cleft by enzymatic degradation is a unique property of the NMJ. In the majority of cases, high affinity uptake mechanisms in neurons and glial cells remove transmitter molecules from the extracellular environment (Kandel et al., 1991). Both of the primary amino acid transmitters in the brain, glutamate and GABA, are carried by specific transporters in neurons and glial cells (Hertz, 1979; Iversen & Kelly, 1975; Nicholls & Attwell, 1990).

While the glutamate uptake inhibitors, dihydrokainate and DL-threo-hydroxyaspartic acid enhance responses to exogenously applied transmitter in hippocampal slices, they have been found to have no effect on synaptic responses mediated by NMDA or non-NMDA receptors (Hestrin et al., 1990). This negative result suggests that transmitter uptake does not contribute to the decay of excitatory synaptic transmission. Alternatively, glutamate uptake mechanisms may be very plentiful near synapses such that the presently available uptake inhibitors may be too weak to affect a great enough fraction of transporters to influence synaptic transmission.

A number of studies suggest that transmitter uptake does influence the time course of inhibitory synaptic transmission in the hippocampus (Dingledine & Korn, 1985; Hablitz & Lebeda, 1985; Rekling, Jahnsen & Laursen, 1990). In general, these studies have found that inhibitors of GABA uptake have no effect on the amplitude of polysynaptic IPSPs but greatly

prolong their decay. While these studies are suggestive, the precise effects of blocking GABA uptake on $GABA_A$- and $GABA_B$- mediated synaptic currents have not been clearly addressed.

III. Methods

Hippocampal slices from adult male guinea pigs were prepared using standard methods (Zalutsky & Nicoll, 1990). Briefly, guinea pigs were anesthetized with Halothane and decapitated. The brain was removed from the skull and placed in an ice cold Ringer solution comprised of (in mM) 125 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1 NaH_2PO4, and 10 glucose and was equilibrated with 95% 0_2 and 5% CO_2 (Carbogen). Hippocampi were quickly dissected free, laid on an agar block, and mounted on a cutting platform with cyanoacrylate (Krazy Glue). The platform was then placed in a slush of Ringer solution in the cutting chamber of a vibrating tissue slicer (Vibratome). Transverse slices were cut at thicknesses of 400 - 500 μm and transferred to a holding chamber where they recovered for at least one hour prior to being placed in a seperate recording chamber. The majority of experiments were performed using a chamber of standard design (Nicoll & Alger, 1981) in which a slice was sandwiched between two nylon nets and superfused with Ringer solution at a rate of 1 - 5 ml/min. The temperature of the chamber could be controlled by a Peltier heating/cooling unit. In some experiments, a second chamber optimized for the laminar flow of the Ringer solution was used. In this chamber, the slice was placed on a cover slip in a plexiglass trough and held in place by a nylon net attached to a platinum frame. The bath solution entered at one end of the trough and was removed by suction at the adjacent end. Experiments were performed at room temperature unless otherwise indicated.

The extracellular superfusing solution was normally identical to the Ringer solution described above. In the majority of experiments examining excitatory synaptic transmission, this solution was supplemented with picrotoxin (50-100 μM) and D-2-amino-5-phosphonovaleric acid (D-APV, 25 μM) to block $GABA_A$ receptors and NMDA receptors, respectively. In some of these experiments the concentration of KCl was increased to 5 mM and the concentrations of $MgCl_2$ and $CaCl_2$ increased to 4 mM. Except where indicated, recordings of inhibitory synaptic transmission were recorded from slices in which NMDA receptors were blocked with D-APV (25 μM) and non-NMDA receptors were blocked with either 6-cyano-7-nitroquinoxaline-2.3-dione (CNQX, 20 μM) or 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM). In the majority of experiments examining iontophoretic responses, tetrodotoxin (0.5-1 μM) was included to block sodium-dependent action potentials.

Patch clamp techniques were used to record from granule cells of the dentate gyrus and pyramidal cells in areas CA1 and CA3 of the hippocampus. Whole-cell recordings were obtained using the "blind" approach (Blanton, Lo Turco & Kriegstein, 1989; Coleman & Miller, 1989). Briefly, under a binocular dissecting microscope, patch pipettes were positioned over the appropriate cell layer and slowly stepped through the slice. Positive pressure was delivered to the patch electrode and the resistance at the tip of the electrode was continuously monitered using an Axopatch 1B amplifier in the current clamp mode. The resistance of the patch electrodes ranged from 3-10 MOhms. When a slight increase in resistance was encountered, the positive pressure was removed and negative pressure was applied to the patch pipette. In the majority of attempts, the resistance at the tip of the electode rose to values in the GOhm range, tentatively indicating a tight seal between the patch electrode tip and a cell membrane. At this point, the Axoclamp was

switched to voltage-clamp mode. While 10 mV voltage steps were applied, further suction caused the rupture of membrane at the tip of the electrode indicated by a large increase in the capacitative current in response to the voltage steps. Access to neurons was confirmed by the presence of rapid inward currents with voltage sensitive thresholds close to those for Na^+-dependent action potentials. Input resistances varied from 100 MOhm - 1 GOhm. Series resistances ranged from as low as 5 MOhms to 50 -75 MOhms and experiments were rejected if the series resistance, determined from voltage steps, was unstable. Once a whole-cell recording was established, outside-out patches could be obtained by pulling the patch pipette out of the slice. All experiments were performed at a holding potential of -80 mV, unless otherwise stated.

Patch electrodes (tip diameter = 2 μm) contained (in mM) either a CsF (110 CsF, 10 CsCl, 10 HEPES, and 10 EGTA, pH 7.3) or Cs-gluconate (117.5 Cs-gluconate, 17.5 CsCl, 8 NaCl, 10 HEPES, 2 MgATP, 0.2 GTP, and 0.2-1 EGTA, pH 7.3) internal solution. In some experiments the Cs-gluconate was replaced by CsCl. In other experiments, a potassium-based internal solution was used and contained (in mM) 130 K-gluconate, 4 NaCl, 10 HEPES, 1 EGTA, 4 MgATP, 1 GTP, pH 7.3.

Extracellular recordings were made from microelectrodes filled with 2 M NaCl and broken at their tips to produce resistances of 3 - 5 MOhms. Field potential recordings of field EPSPs and population spikes were made from recording electrodes placed in either the dendritic region or cell layer of slices, respectively.

In many experiments drugs were bath applied to the entire slice. In most cases, drugs were dissolved in distilled water as concentrated stock solutions and added directly to the superfusing solution. Stock solutions of

0.5 M aniracetam or piracetam and 20 mM CNQX or DNQX were dissolved in dimethylsulphoxide (DMSO) and added directly to the superfusing medium. DMSO alone, diluted to 0.6% in the medium had no effects.

Transmitter agonists were applied to individual cells in the slice by iontophoresis. Agonists were delivered to outside-out patches using pressure ejection or rapid perfusion techniques.

Agonists applied by iontophoresis were delivered from glass microelectrodes controlled by a WPI Microiontophoresis Programmer. Single or double barrelled iontophoretic electrodes were filled with either glutamate (250 mM, pH 8), quisqualate (10 mM in 150 mM NaCl, pH 8), kainate (5 mM in 150 mM NaCl, pH 8), domoate (5 mM in 150 mM NaCl, pH 8), GABA (1 M in distilled H_20 or 50 mM in 150 mM NaCl, pH 4) or the GABA analog 4,5,6,7-tetrahydroisoxazolo{5,4-c} pyridine-3-ol (THIP, 50 mM in 150 mM NaCl, pH 4).

GABA (25 μM dissolved in the superfusion solution) was applied to outside-out patches by pressure ejection from a broken pipette using a Picospritzer II (General Valve).

To measure glutamate desensitization, outside-out patches were maneuvered within the laminar flow slice chamber to an apparatus constructed for the rapid application of agonists (Franke, Hatt & Dudel, 1987; Trussell & Fischbach, 1989). Briefly, this device consisted of a piezoelectric bimorph element (Vernitron) attached to a segment of theta tubing with a tip diameter of ≈250 μm (Fig. 1). Perfusing solutions were gravity-fed to each compartment of the theta tubing. The tip of the patch electrode was positioned ≈100 μm from the tip of the theta tubing, close to the sharp boundary that formed between the two flowing solutions. Solution exchange was achieved by rapidly driving the boundary across the electrode tip with a

voltage pulse to the piezoelectric element, remotely triggered at rates of 0.1 - 0.3 Hz. The speed of solution exchange was assessed by measuring the change in current generated by the shift in liquid junction potential at the tip of an open patch electrode in response to the rapid exchange of the normal perfusing solution to a solution diluted 10-fold with distilled water. Open-tip current measurements showed that the fastest solution exchanges were complete within 2 ms.

Data were recorded and analyzed on a IBM PC clone (Dell) using software provided by Drs. S. Hestrin and D. Perkel. In some experiments, data were recorded on video tape with a VCR (Vetter) for later analysis. Some records of cell membrane current were plotted on a Gould chart recorder. Illustrated traces are averages of 3 - 5 responses, except where indicated. Results are presented as mean ± SEM and significance was assessed using Student's t-test.

Aniracetam was generously supplied by Dr. P. Sorter, Hoffman-La Roche. SKF 89976A was a gift from Dr. W. Karbon, Nova Pharmaceutical Corporation, and CGP 35348 was generously provided by Drs. H. Schröter and L. Maître, Ciba-Geigy.

The Role of Channel Kinetics in Governing the Time Course of Fast Excitatory Transmission

Introduction

A general property of neurotransmitter receptor-channels is that in the continuous presence of agonist the response rapidly diminishes. Although this desensitization, which results from a conformational change in the receptors, is readily demonstrated for a number of neurotransmitters including acetylcholine (Katz & Thesleff, 1957; Magleby & Pallotta, 1981), gamma-aminobutyric acid (Akaike, Inoue & Krishtal, 1986), glycine (Krishtal, Osipchuk & Vrublevsky, 1988), serotonin (Yakel & Jackson, 1988), and glutamate (Dudel, Franke & Hatt, 1990; Franke et al., 1987; Kiskin et al., 1986; Mayer, 1989; Mayer & Vyklicky, 1989; Tang et al., 1989; Trussell & Fischbach, 1989; Trussell et al., 1988), a physiological role for desensitization in synaptic transmission has not been established. Rapid perfusion experiments with the non-NMDA type of glutamate receptor indicate that this receptor desensitizes extremely quickly in the presence of glutamate (Kiskin et al., 1986; Mayer, 1989; Mayer & Vyklicky, 1989; Tang et al., 1989; Trussell & Fischbach, 1989) with a time course similar to that of glutamate-mediated synaptic responses. This similarity has led to the proposal (Tang et al., 1989; Trussell & Fischbach, 1989) that the duration of fast excitatory transmission in the brain is limited by receptor desensitization, rather than by the unbinding rate of transmitter, as is the case at the NMJ.

Recent studies have indicated that the time course of the slow NMDA receptor-mediated EPSC can be accounted for by the slow kinetics of the

channels underlying the synaptic current (Hestrin et al., 1990; Lester et al., 1990). Although the high Q_{10} of the non-NMDA receptor-mediated EPSC (Hestrin et al., 1990) suggests that properties other than diffusion determine the time course of this response, little else is known about the factors governing the decay of the synaptic current. In this study, we have examined the role of channel kinetics in determining the time course of fast excitatory transmission in the hippocampus.

To address this question, we have studied the actions of aniracetam, a drug belonging to a class of compounds (Fig. 1) that are classified as "nootropic", or cognitive-enhancing agents (Heise, 1987). While some of these compounds have been suggested to improve cognitive functions in rats and humans (Martin, Cumin, Aschwanden, Moreau, Jenck & Haefely, 1992; Mondadori, Petschke & Häusler, 1989), the actions of these drugs at the cellular or molecular level is not known. In a recent study, aniracetam has been shown to selectively enhance quisqualate receptor-mediated responses both in *Xenopus* oocytes injected with brain messenger RNA and in hippocampal pyramidal cells (Ito, Tanabe, Kohda & Sugiyama, 1990). We have examined, more closely, the mechanism for its selective action and its effects on synaptic transmission in the hippocampus. We find that aniracetam enhances glutamate-evoked currents in whole-cell recordings and, in macroscopic current recordings in outside-out patches, strongly reduces glutamate receptor desensitization. Aniracetam also enhances the time course of responses to brief applications of glutamate, that are determined by the transmitter unbinding rate. In addition, aniracetam selectively prolongs the time course and increases the peak amplitude of fast synaptic currents. These findings indicate that aniracetam slows the kinetics of fast synaptic transmission and are consistent with the proposal that both receptor

desensitization and unbinding of transmitter could determine the rate of decay of the non-NMDA receptor-mediated EPSC.

Results

Bath application of aniracetam caused a dramatic enhancement in the size of glutamate-evoked responses measured with whole-cell recording techniques from neurons in the hippocampal slice preparation (n=10) (Fig. 2). The effect, which was observed in CA1 and CA3 pyramidal cells as well as dentate granule cells, developed as rapidly as the solution exchange in the recording chamber (1 min) and the response recovered quickly following washout of the drug. On average the glutamate response was enhanced 209 ± 35%.

The ability of aniracetam to enhance these responses in cells dialyzed with a fluoride-containing internal solution, which would be expected to disrupt many metabolic processes (Zalutsky & Nicoll, 1990), as well as its rapid action in cell-free patches (see below), favors an allosteric mechanism of action of the drug. Piracetam, a structurally-related member of the nootropic class of drugs (Fig. 1) (Heise, 1987), had no effect on glutamate responses, although these responses were potentiated by the subsequent administration of aniracetam (n=3). Therefore, the enhancing effect is not a common property of all nootropic drugs.

We have confirmed in the hippocampal slice the finding of Ito et al. in *Xenopus* oocytes injected with rat brain mRNA (Ito et al., 1990) that aniracetam enhances the action of quisqualate (n=12) but not that of kainate (n=6) (Fig. 3) or domoate, a kainate-like agonist (n=9). The finding by others that quisqualate can desensitize kainate responses (Kiskin et al., 1986; Patneau & Mayer, 1991) and that glutamate receptors expressed from cDNA clones can respond to both quisqualate and kainate (Boulter et al., 1990; Keinånen et al., 1990) suggests that a single receptor responds to both of these agonists. Since the responses in CNS neurons to quisqualate and glutamate desensitize

whereas those to kainate and domoate do not (Kiskin et al., 1986; Kiskin, Krishtal & Tsyndrenko, 1990; Patneau & Mayer, 1991), we considered the possibility that the selective action of aniracetam might be due to a reduction of receptor desensitization.

To examine this possibility, outside-out patches were made from the somata of pyramidal and granule cells in the hippocampal slice. Drugs were rapidly delivered to the patch for 100-200 ms via an apparatus that allowed the switching of perfusing solutions within 2 ms (Fig. 4). The peak amplitudes of responses to glutamate ranged from 25 to 200 pA. These sizable macroscopic currents suggested a high density of somatic glutamate receptors. In agreement with studies in cultured hippocampal neurons (Kiskin et al., 1986; Mayer, 1989; Mayer & Vyklicky, 1989; Tang et al., 1989), the responses to glutamate and quisqualate decreased rapidly in the continued presence of agonist (Fig. 5). The responses to glutamate desensitized with time constants as short as 4.5 ms and with an average of 16.8 ± 8 (n=9), values which are probably limited by the speed of the solution change. In contrast, responses to kainate were maintained for the duration of agonist application (Fig. 5).

The time course of the desensitization of the responses to glutamate in outside-out patches was quite similar to the time course of the fastest excitatory synaptic currents in the slice (Fig. 6). The time constant of decay of EPSCs were quite variable, ranging from 4 ms to many tens of milliseconds. Long decay time constants were usually associated with synaptic currents that were evoked far from the soma of the recorded neuron or from recordings in which the series resistance was high (> than 20 MOhms). We have assumed that the fastest currents reflect the true decay of the EPSC and that the slowly rising and decaying currents represent responses that have

been electrotonically filtered due to improper voltage clamp (Hestrin et al., 1990).

We next examined the effect of aniracetam on the responses to long (≈200 ms) pulses of glutamate in outside-out patches. In the presence of aniracetam, the rate of desensitization of these responses was markedly slowed (215 ± 43%, n=9) and the peak amplitude of the responses increased (51 ± 11%, n=10) (Fig. 7A). In contrast, responses to kainate were unaffected (Fig. 7B).

This action of aniracetam on macroscopic glutamate currents is similar to the effect of lectins on glutamate responses in cultured cells (Mayer & Vyklicky, 1989; Zorumski, Thio, Clark & Clifford, 1990). In these studies it has been suggested that the selective effect of lectins on the rapid inactivation of the responses to glutamate, AMPA, and quisqualate, coupled with their lack of effect on the responses to kainate, indicate that their major action is to reduce receptor desensitization.

Given the dramatic effects of aniracetam on responses to glutamate we next examined its effects on synaptic currents at Schaffer collateral-commissural synapses in CA1 (n=5), mossy fiber synapses in CA3 (n=3) and perforant path synapses in the dentate gyrus (n=5). Aniracetam had two very consistent effects at these synapses. It prolonged the rate of decay and increased the peak amplitude of the EPSC (Fig. 8). In a number of cells, the average increase in the decay time constant was 90 ± 9% (n=10) while the peak current increased 116 ± 14% (n=10). There was no obvious difference in the effect of aniracetam on the different types of synapses in the hippocampus. The two effects of aniracetam had similar dependencies on concentration and similar time courses of action, suggesting that they may be due to a common mechanism of action. These effects on synaptic currents

would be expected to greatly augment synaptic potentials recorded in current clamp, as reported previously (Ito et al., 1990).

We also examined the actions of aniracetam on synaptic transmission using extracellular recording techniques. A recording electrode was placed in the dendritic region of the slice to measure a field EPSP, representing the activation of a large number of synapses from a population of neurons. Similarly, if the stimulus strength is high enough, a recording electrode placed in the cell layer detects a population spike reflecting the orthodromic action potentials evoked in a population of cells. In contrast to its effects on synaptic responses measured with whole-cell recording, the effects of aniracetam on the field EPSP were much less dramatic. Aniracetam had little or no effect on the slope of the rising phase of the EPSP (Fig. 9A). This result was suprising since the initial slope of the field EPSP is clearly increased by other manipulations which are known to enhance synaptic strength. The drug did, however, prolong the decay of the field EPSP and often caused a slight increase in the peak amplitude of the synaptic potential. Aniracetam had a much more obvious effect on the population spike. Aniracetam caused a dramatic increase in the amplitude of the spike (Fig. 9B), indicating that the cells were more likely to reach threshold in response to synaptic stimulation.

In another series of experiments, we addressed the specificity of the action of aniracetam in the slice. Piracetam, which had no effect on glutamate responses (see above), also had no effect on EPSCs (n=4) (Fig. 10A). Another nootropic compound, oxiracetam (Heise, 1987), was similarly ineffective (n =2). These results and the previous experiments studying glutamate responses in the slice suggests that the enhancing effect of aniracetam is not a common property of all nootropic drugs.

Synaptically-released glutamate acts on both N-methyl-D-aspartate (NMDA) and non-NMDA receptors, which are co-localized at single excitatory synapses (Bekkers & Stevens, 1989; Hestrin, Perkel, Sah, Manabe, Renner & Nicoll, 1990). In contrast to the enhancing action of aniracetam on non-NMDA receptor-mediated EPSCs, no effect was detected on the NMDA component of the EPSC. This is shown in Fig. 10B, in which aniracetam was first shown to exert its normal effect at a holding potential of -80mV. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was then added to the superfusion medium to block the non-NMDA component of the EPSC and the holding potential was shifted to +50mV to alleviate the Mg^{++} block of the NMDA synaptic current. Aniracetam had no effect on the NMDA receptor-mediated EPSC, although post-tetanic potentiation (PTP), which increases transmitter release (Zucker, 1989), clearly enhanced this current (n=4). Since manipulations that enhance transmitter release typically potentiate both components of the EPSC (Hestrin et al., 1990; Kauer, Malenka & Nicoll, 1988), the lack of effect of aniracetam on the NMDA component favors a post-synaptic mechanism for its action on the non-NMDA component.

To further characterize the site at which aniracetam alters the EPSC, we examined its action on miniature EPSCs (mEPSCs) recorded in the presence of tetrodotoxin to block action potential-dependent synaptic events. These synaptic responses represent the spontaneous exocytosis of synaptic vesicles from presynaptic terminals and modifications in their strength are most easily accounted for by postsynaptic mechanisms (Manabe et al., 1992; Redman, 1990). In the presence of TTX , mEPSCs could clearly be distinguished using whole-cell recording techniques in hippocampal cells (Fig.11). These events typically occurred at a frequency of ≈ 1 Hz. To increase the frequency of these events, a hyperosmotic sucrose solution was applied via puffer pipette onto

the dendrites of granule and pyramidal cells (Bekkers & Stevens, 1989). Aniracetam prolonged the time course of mEPSCs in all cells examined and in four of the six cells caused an increase in their peak amplitude (Fig. 12).

The effects of aniracetam on macroscopic responses to glutamate in outside-out patches along with the action of the drug on synaptic responses are consistent with the proposal that receptor desensitization limits the decay of excitatory synaptic currents. Recently, results identical to ours were reported from two different groups that concluded that aniracetam slowed desensitization (Tang, Shi, Katchman & Lynch, 1991; Vyklicky, Patneau & Mayer, 1991). However, it has been suggested that similar results could also be accounted for by an action of aniracetam on channel open time, distinct from an effect on receptor desensitization (Vyklicky et al., 1991).

To differentiate between an effect on desensitization versus the channel closing rate, we examined the effect of aniracetam on paired pulses of glutamate applied to outside-out patches. In these experiments, the peak response to a second pulse of glutamate was smaller than the response to a preceding pulse. This depression of the response was due to the fact that the desensitization induced by the first pulse "inactivated" some fraction of channels in the patch, rendering them unresponsive to the glutamate applied in the second pulse. Fig. 13A shows the responses to pairs of applications of quisqualate delivered at interpulse intervals of 100 - 600 ms. As reported previously using cultured neurons (Trussell & Fischbach, 1989), the recovery from desensitization was slow, taking hundreds of milliseconds to completely recover. Fig. 13B shows the effect of aniracetam on paired pulses of quisqualate in one patch. While aniracetam prolonged the decay of the individual responses to quisqualate, it also reduced the depression of the peak amplitude of the second pulse. The results before and after treatment

with the drug in this experiment are plotted graphically and (Fig. 13C) show that aniracetam clearly reduced the desensitization of the second pulse at all interpulse intervals. This experiment suggests that the actions of aniracetam are, at least in part, due to a reduction in receptor desensitization.

In contrast to the slow channel kinetics of the NMDA receptors (Hestrin, 1992b; Hestrin et al., 1990; Lester et al., 1990), recent studies of non-NMDA receptors in the hippocampus (Jonas & Sakmann, 1992) and visual cortex (Hestrin, 1992a) indicate that the channel closing rate following the removal of agonist is faster than the rate of onset of desensitization. We observed similar results when very brief pulses of glutamate were appled to outside-out patches. As shown in Figure 14A, the response to a 4 ms pulse of glutamate typically decayed faster than the desensitization of the response to a long pulse of agonist. Since the glutamate pulse is switched off before the receptors begin to desensitize, the decay of these brief responses should be governed by the dissociation rate of the transmitter (channel closing rate).

It has been suggested that due to free diffusion the lifetime of transmitter in the synaptic cleft is brief and the transmitter concentration should decay with a time constant of about 1 ms (Eccles & Jaeger, 1958). If this were the case, and transmitter was effectively cleared from the synapse before receptor desensitization could begin, the ability of aniracetam to greatly prolong the EPSC would best be explained by an effect on the channel closing rate. Indeed, Hestrin has recently demonstrated that aniracetam prolongs the time course of responses to brief pulses of glutamate, which decay faster than the rate of desensitization (Hestrin, 1992a). In agreement with these findings, we have found that aniracetam also prolonged the response to short pulses of glutamate (Fig. 14B) in patches from hippocampal neurons.

Long-term potentiation (LTP) is an intensely studied phenomenon in which brief repetitive synaptic stimulation paired with postsynaptic depolarization results in a long-lasting enhancement of synaptic transmission (Madison et al., 1991). We have also examined whether LTP and aniracetam might share a common mechanism in their enhancement of synaptic strength. In one series of experiments we studied the effect of LTP on the kinetics of the synaptic current. In contrast to the effect of aniracetam on the time course of the EPSC, LTP had no obvious effect on the decay of the synaptic current (n=6) (Fig. 15).

It has recently been reported that the action of aniracetam on the amplitude of field EPSPs is less in pathways expressing LTP (Staubli, Kessler & Lynch, 1990; Xiao, Staubli, Kessler & Lynch, 1991). In another series of experiments we examined the enhancing effect of aniracetam on naive synapses versus potentiated ones. Fig. 16 shows an example of an experiment with whole-cell recording in which a comparison between LTP and the action of aniracetam on the amplitude of the EPSC has been made in the same cell. In this experiment the responses to two independent pathways converging onto the same cell were recorded. Tetanic stimulation to one pathway, paired with postsynaptic depolarization, caused a large increase in the size of the EPSC. However, when the stimulus strength of the pathway expressing LTP was reduced so that the peak amplitude of the synaptic response was the same as that in the independent control pathway, aniracetam had similar effects on both pathways. In 6 cells aniracetam increased the size of the EPSC in the control pathway 108 ± 19% and in the pathway expressing LTP, aniracetam increased the EPSC 126 ± 19%. The difference was not significant. These results indicate that aniracetam and LTP apparently enhance synaptic strength through different mechanisms.

Discussion

We have used rapid perfusion techniques and outside-out membrane patches from hippocampal slices to elucidate the mechanism for the enhancing action of aniracetam on glutamate responses. Aniracetam selectively enhanced responses to glutamate and quisqualate in the slice, but had no effect on the responses to kainate. Since the responses to glutamate and quisqualate desensitize and responses to kainate do not, we reasoned that aniracetam exerted its selective action via a reduction of receptor desensitization. In support of this hypothesis, aniracetam markedly slowed the rate of desensitization of macroscopic responses to glutamate in outside-out patches.

Given the dramatic action of aniracetam in outside-out patches, we therefore used this drug to test the proposal (Trussell & Fischbach, 1989) that glutamate receptor desensitization might limit excitatory synaptic transmission. Indeed, aniracetam produced nearly a doubling in both the decay time constant and amplitude of EPSCs.

The ability of aniracetam to increase the amplitude of both the responses to the rapid application of glutamate and EPSCs might arise from a number of mechanisms. One possibility is that a fraction of glutamate receptors might rapidly enter a desensitized state during the onset of the glutamate response and the rising phase of the EPSC. Therefore, the peak of these macroscopic currents would be limited by desensitization. Another possibility is that the peak of evoked EPSCs is enhanced by aniracetam because of the asynchronous release of transmitter from many activated synapses. If vesicular release continues during the rising phase of the evoked EPSC, then the prolongation of individual synaptic responses (mEPSCs) would cause a greater degree of "temporal summation" at the peak of the

evoked synaptic current. In support of this arguement, we noticed that, compared to its action on mEPSCs, aniracetam had a greater effect on the amplitude of large, evoked EPSCs. Alternatively, it has been shown that glutamate concentrations in the same range as that detected in the extracellular fluid (2 - 4 μM) (Lerma, Herranz, Herreras, Abraira & Martin Del Rio, 1986; Sah, Hestrin & Nicoll, 1989) can desensitize glutamate responses and mEPSCs (Trussell & Fischbach, 1989). Thus, the increase in amplitude of the EPSC might reflect the removal of background desensitization thereby increasing the number of functional receptors.

However, the effects of aniracetam on another measure of synaptic strength, the slope of the field EPSP, argue against a role for background desensitization. The drug caused little or no detectable increase in the slope of the EPSP, although it generally prolonged its decay. In contrast, the slope does increase in response to an increase in stimulus strength (c.f. Fig 16), which, by adding more active afferent fibers to the response, is a simple manipulation that increases the number of receptors contributiong to the EPSP. While aniracetam had little effect on the slope of the field EPSP, a prolongation of the decay of the conductance underlying the EPSP should enhance the depolarizing input to unclamped neurons and increase the likelihood that evoked synaptic responses would cause neurons to fire action potentials. In support of this hypothesis, the population spike was greatly enhanced in the same experiments in which the slope of the field EPSP was not affected. Thus, aniracetam does enhance synaptic strength in the hippocampus, but does so without a large steady-state increase in the number of available synaptic receptors.

While at the macroscopic level aniracetam appeared to act primarily by blocking desensitization, it cannot be ruled out that at the single channel level

aniracetam might alter channel kinetics by an action independent of desensitization. We attemped to address the precise molecular process affected by aniracetam by studying its actions on the receptor desensitization underlying the paired pulse depression of glutamate responses in outside-out patches. We found that aniracetam clearly reduced the desensitization of the second of a pair of glutamate pulses. However, in agreement with results in the visual cortex (Hestrin, 1992a), aniracetam also prolonged the time course of the responses to brief pulses of glutamate, which decays faster than the rate of desensitization and is primarily limited by the rate of channel closure. These results suggest that aniracetam can both slow the transition of the receptors into a desensitized state and prolong the channel open time.

At both the ACh receptors of the NMJ and NMDA receptors, the decay of the synaptic current is due to channel kinetics that are much longer than the lifetime of free transmitter in the synaptic cleft (Hestrin et al., 1990; Lester et al., 1990; Magleby & Stevens, 1972b). Does receptor desensitization contribute to the time course of non-NMDA receptor synaptic currents? Clements and colleagues have recently estimated the time course of free glutamate in the cleft by the kinetic analysis of the displacement of a competitive antagonist from NMDA receptors during synaptic transmission (Clements, Lester, Tong, Jahr & Westbrook, 1992). To fit their data, Clements et al. propose that the glutamate concentration peaks at 1.1 mM and decays with a time constant of 1.2 ms. This rapid removal of transmitter from the cleft favors transmitter dissociation from the non-NMDA receptor as the major factor determining the decay of the postsynaptic current. However, it does not exclude the possibility that receptor desensitization could contribute to the decay of the EPSC (Clements et al., 1992).

The actions of aniracetam on synaptic strength differ from concanavalin A and wheat germ agglutinin (WGA), lectins that reduce glutamate receptor desensitization, but do not appreciably prolong synaptic responses in cultured neurons (Mayer & Vyklicky, 1989; Vyklicky et al., 1991). This lack of effect might be due to the poor access of lectins to the synaptic region. Indeed, we have found that concanavalin A and WGA have no effect on glutamate or synaptic responses recorded from pyramidal cells in the slice (not shown). Alternatively, the lectins might exert their sole effect on glutamate receptor desensitization, in contrast to aniracetam which can also prolong channel open time. If this were the case, it would suggest that desensitization does not contribute to the decay of the EPSC.

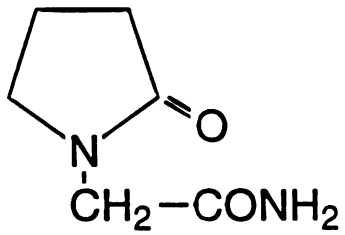
Since aniracetam causes a large enhancement of EPSCs we examined the possibility that it might share the same mechanism as long-term potentiation. However, in contrast to the action of aniracetam, no obvious change in the decay time constant of the EPSC was seen with LTP. In addition, while we cannot rule out subtle interactions between the action of aniracetam and LTP (Staubli et al., 1990; Xiao et al., 1991), it is clear from our results with whole-cell recording that the major action of aniracetam on synaptic strength is distinct from LTP. In agreement with our results, Asztely et al. (Asztely, Hanse, Wigstrom & Gustafsson, 1992) have reported a similar lack of interaction between aniracetam and LTP in the hippocampus.

In this study, we have described the allosteric modulation of glutamate receptors by aniracetam. Aniracetam modifies both the channel closing rate and the entry of the receptor into a desensitized state. Furthermore, we find that aniracetam enhances the amplitude and slows the decay of the EPSC. These results provide further evidence that, like the NMJ, the time course of

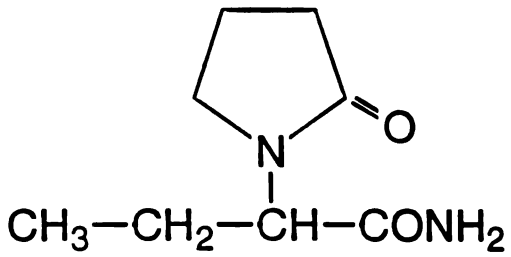
fast synaptic transmission in the brain is determined by the intrinsic properties of postsynaptic receptors.

Figures

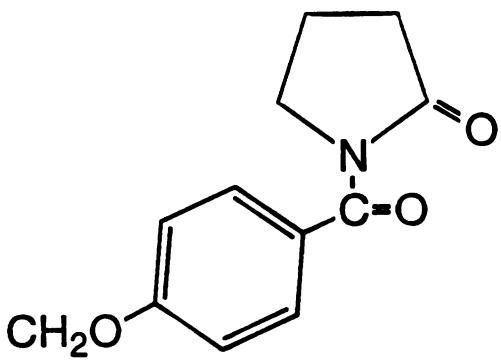
Fig. 1 Chemical structures of the 2-pyrol-lidinone compounds belonging to the "nootropic" class of drugs.



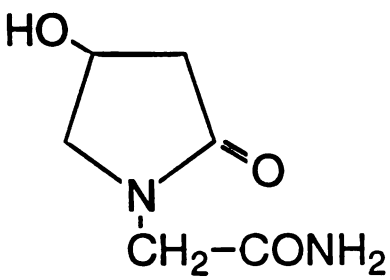
Piracetam



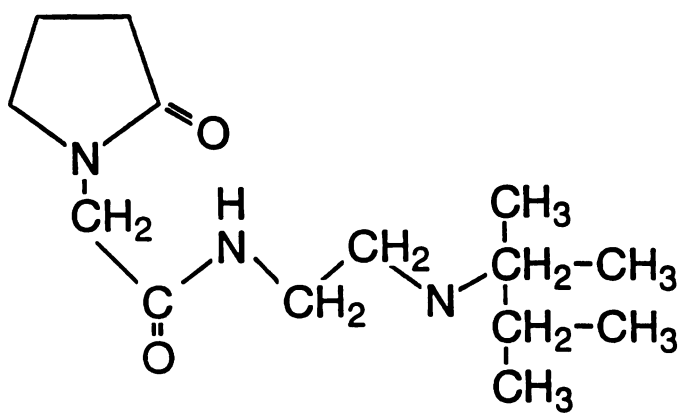
Etiracetam



Aniracetam



Oxiracetam



Pramiracetam

Fig. 2 Aniracetam enhances the response to glutamate in the hippocampal slice A, a chart record of membrane current from a voltage-clamped CA1 pyramidal cell (Vm = -80 mV). Brief iontophoretic pulses of glutamate (70 nA) were applied from an electrode positioned in s. radiatum. Aniracetam (2 mM), added to the bathing medium during the time indicated by the bar, increased the size of the glutamate-induced inward current. B, glutamate responses from the same cell before, during , and after drug application are displayed on a faster time scale. The bar marks the duration of glutamate iontophoresis.

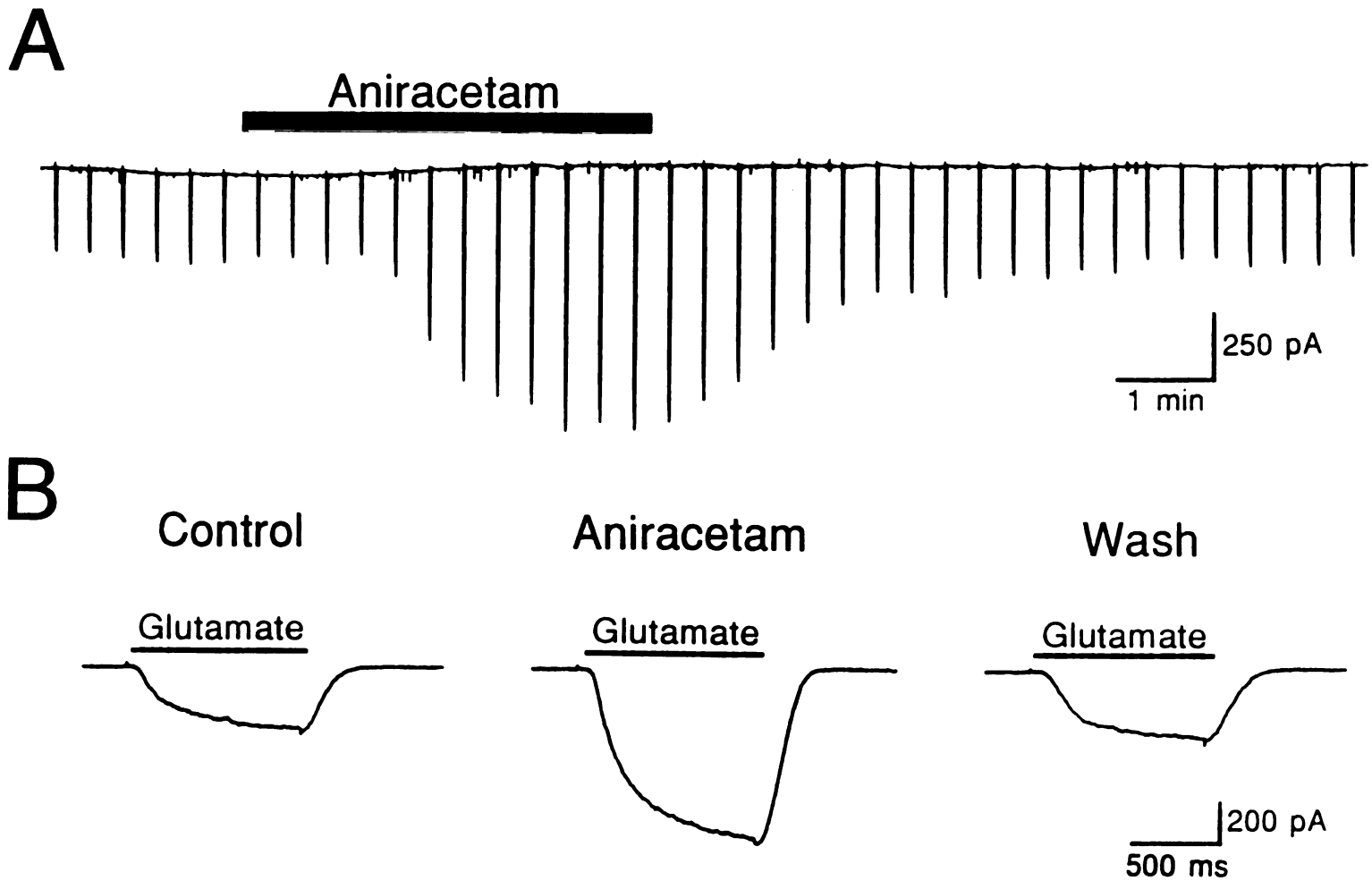

A
Aniracetam
250 pA
1 min
B
Control
Aniracetam
Wash
Glutamate
Glutamate
Glutamate
200 pA
500 ms

Fig. 3 Aniracetam selectively enhances the response to quisqualate but not kainate in patch-clamped hippocampal neurons. Membrane current was recorded from a CA3 neuron and quisqualate (▼, 50 nA/2 s pulse) and kainate (● , 200 nA/4 s pulse) were applied from a double-barrelled iontophoretic electrode placed in s. lucidum. Aniracetam (1 mM) caused a marked increase in the response evoked by quisqualate, but had no effect on the kainate response.

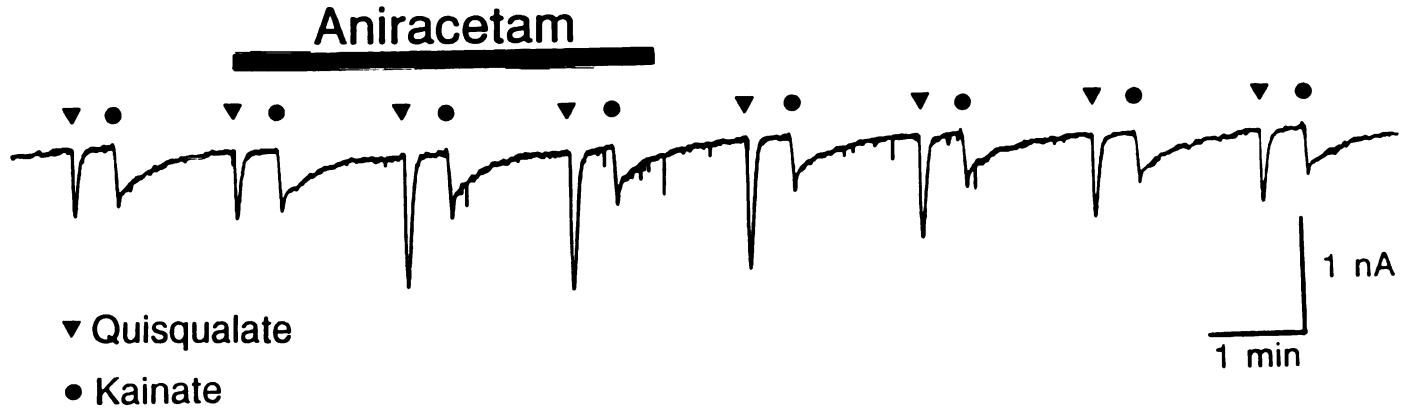


Fig. 4 Schematic diagram of rapid perfusion technique and a demonstration of the speed of solution exchange. A, schematic diagram of perfusion device. A piezoelectric bimorph element rapidly drives the boundary formed between two flowing solutions across the patch electrode tip (see Methods). B, open tip current measurement of the speed of solution exchange. In this experiment, the change in current represents the shift in liquid junction potential at the tip of an open patch electrode in response to the rapid exchange of the normal perfusing solution to a solution diluted 10-fold with distilled water. C, onset of the open-tip current plotted on a faster time scale. Solution exchange was complete within 1 ms.

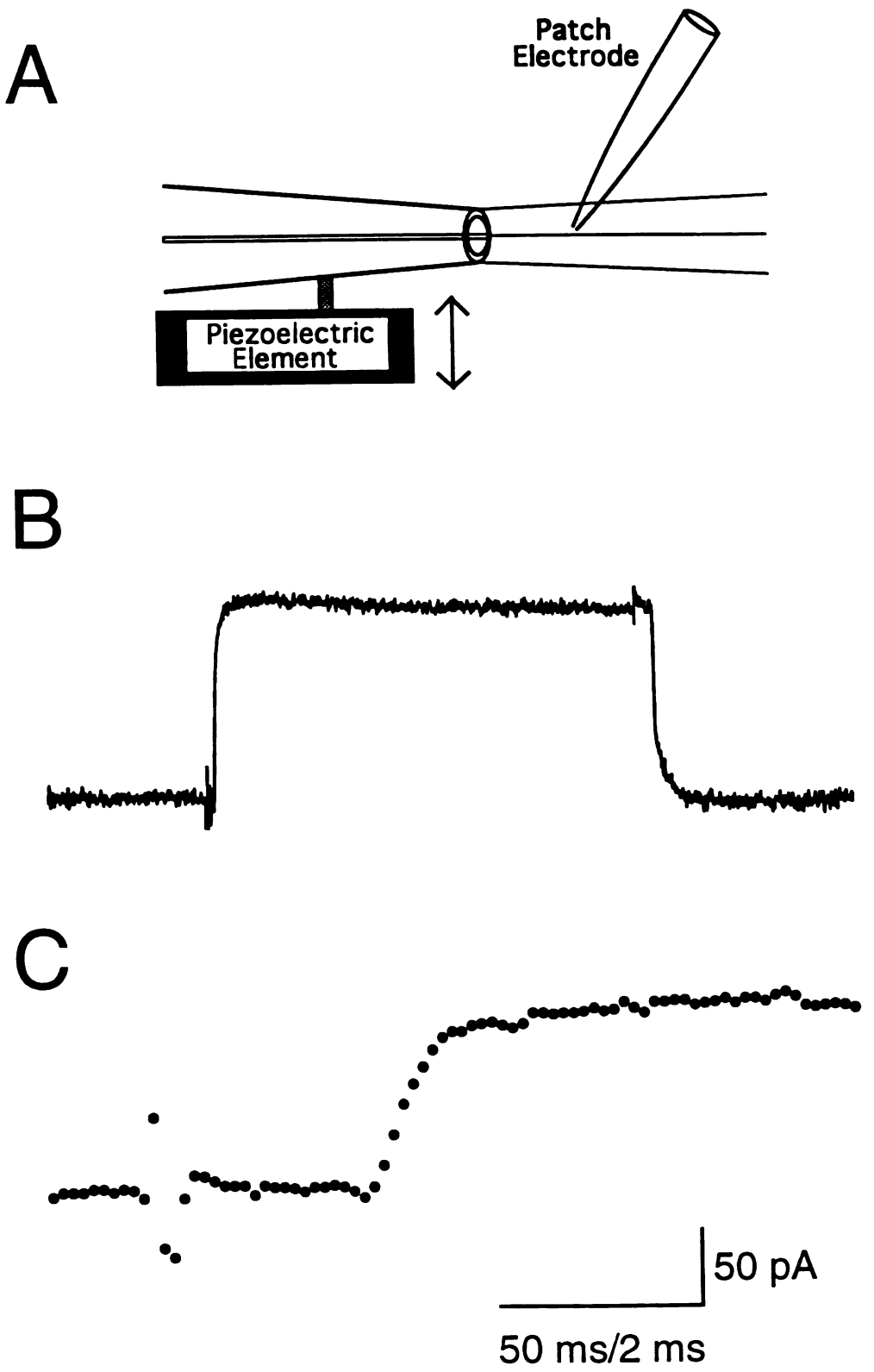

A
Patch
Electrode
Piezoelectric
Element
B
C
50 pA
50 ms/2 ms

Fig. 5 Characterization of the responses to the non-NMDA receptor agonists glutamate, quisqualate, and kainate in outside-out patches. Glutamate (1 mM), quisqualate (100 μM), and kainate (2 mM) were rapidly applied to outside-out patches from three CA1 neurons (Vh =-70 mV). Glutamate and quisqualate, applied for 500 ms, caused the generation of rapidly activating inward currents that quickly decayed in the continued presence of agonist. In contrast, responses to kainate (100 ms application) activated rapidly but were maintained for the duration of agonist exposure.

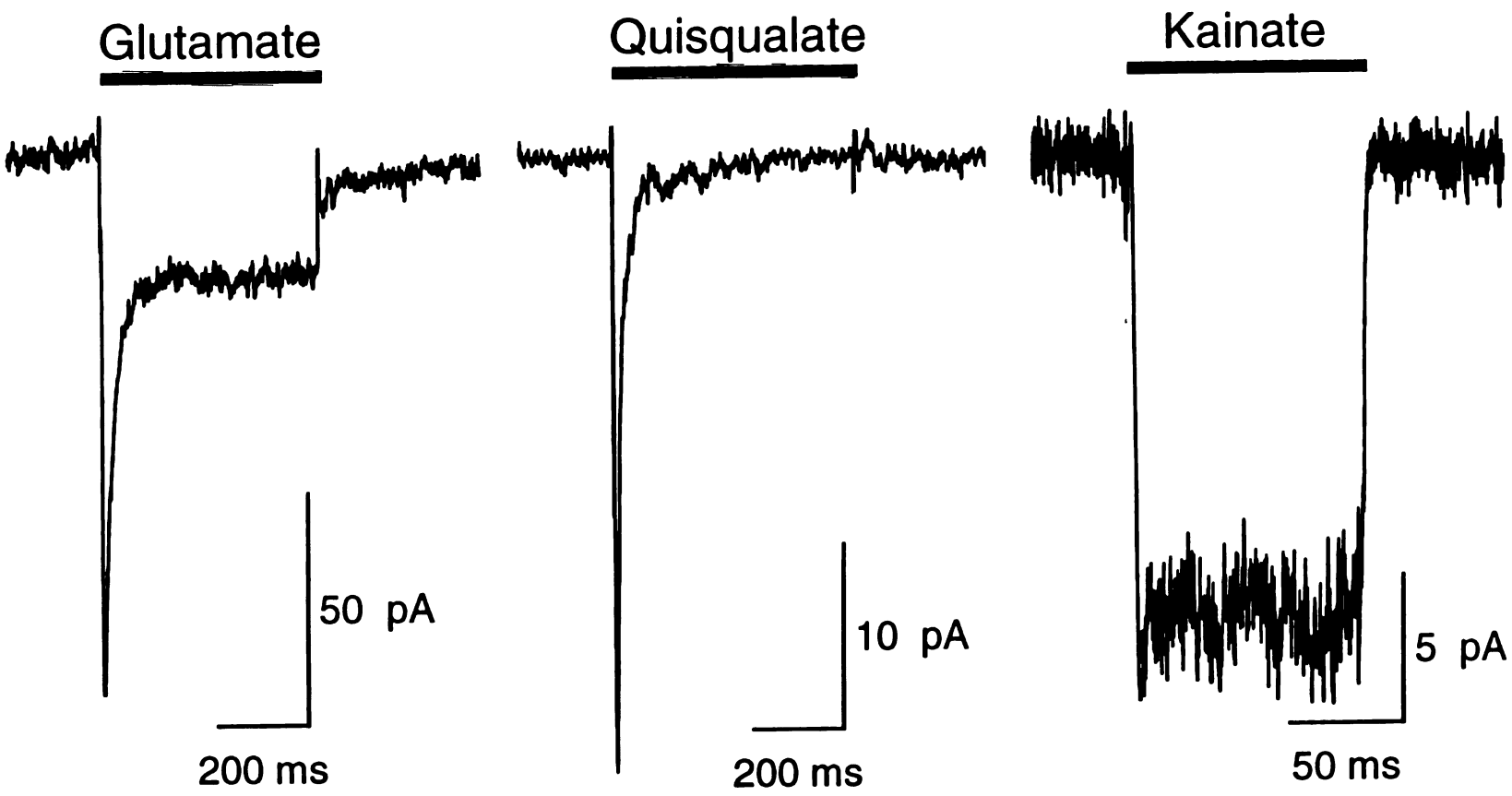
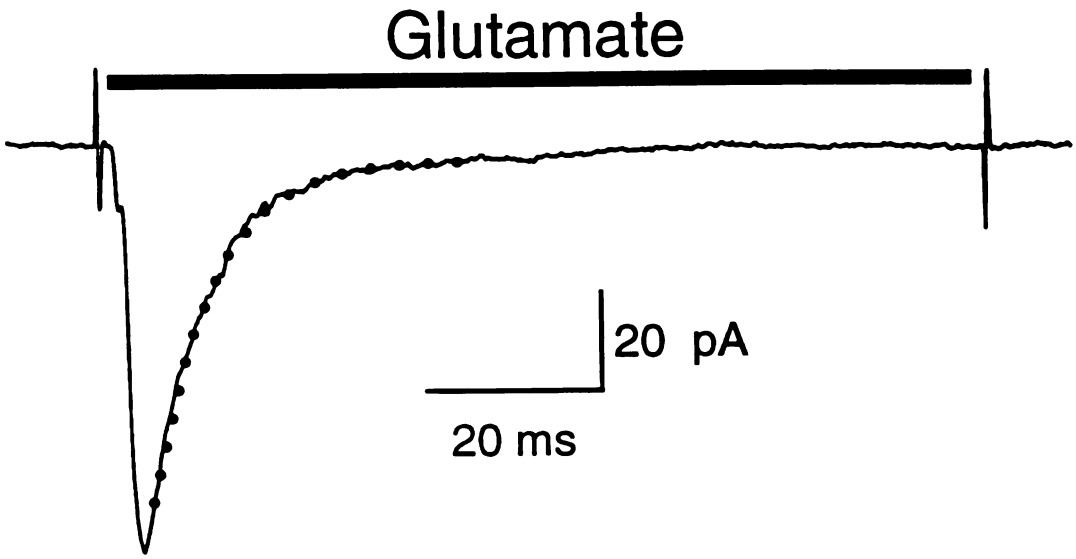

Glutamate
50 pA
200 ms
Quisqualate
10 pA
200 ms
Kainate
5 pA
50 ms

Fig. 6 Time course of glutamate receptor desensitization is similar to the time course of excitatory postsynaptic currents (EPSCs) in the hippocampus. *Top,* response to 1 mM glutamate (100 ms) in a granule cell outside-out patch (Vh=-70 mV). The onset of desensitization in this patch is fit by a single exponential curve (filled circles) with a tau value of 6.3 ms. *Bottom,* an EPSC evoked in a granule cell by stimulation in the perforant path (Vh=-80 mV). The decay of the EPSC is fitted by a single exponential (circles) with a tau value of 6.1 ms.


Glutamate
20 pA
20 ms

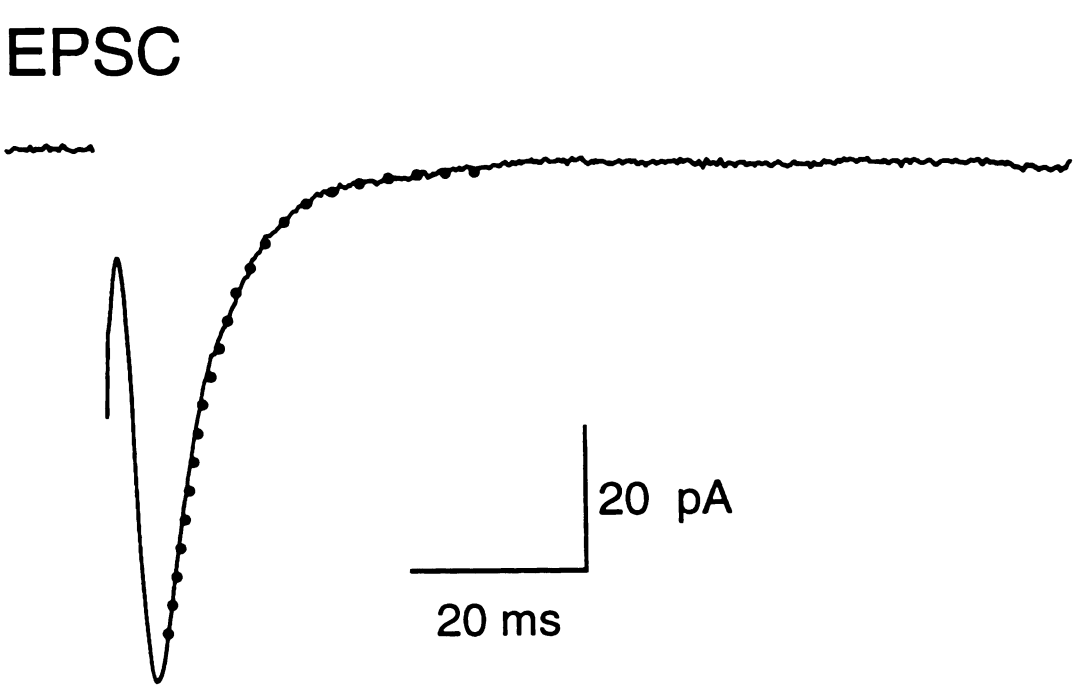

EPSC
20 pA
20 ms

Fig. 7 Aniracetam reduces the desensitization of responses to glutamate but does not affect the response to kainate. A, responses to a 500 ms glutamate application in a CA1 outside-out patch. Macroscopic currents were recorded in the absence and presence of aniracetam (2 mM) . Glutamate (2 mM) was applied for the time marked by the bar. Aniracetam caused a fourfold reduction in the rate of desensitization and increased the steady-state current level to a much greater extent than the peak response. B, aniracetam (2 mM) had no effect on the response to the rapid perfusion of kainate (2 mM, n=2). The response in aniracetam is superimposed over the control response.

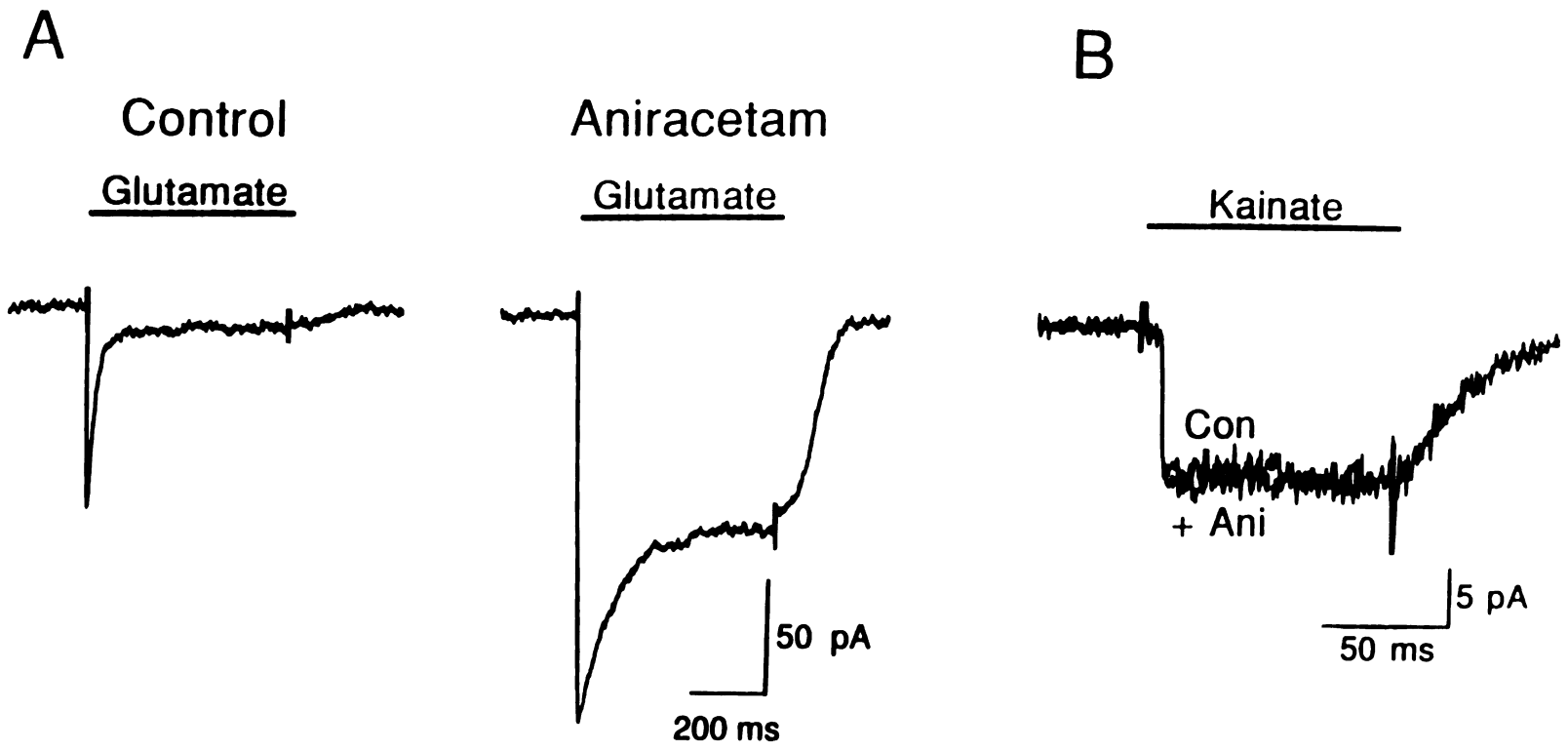
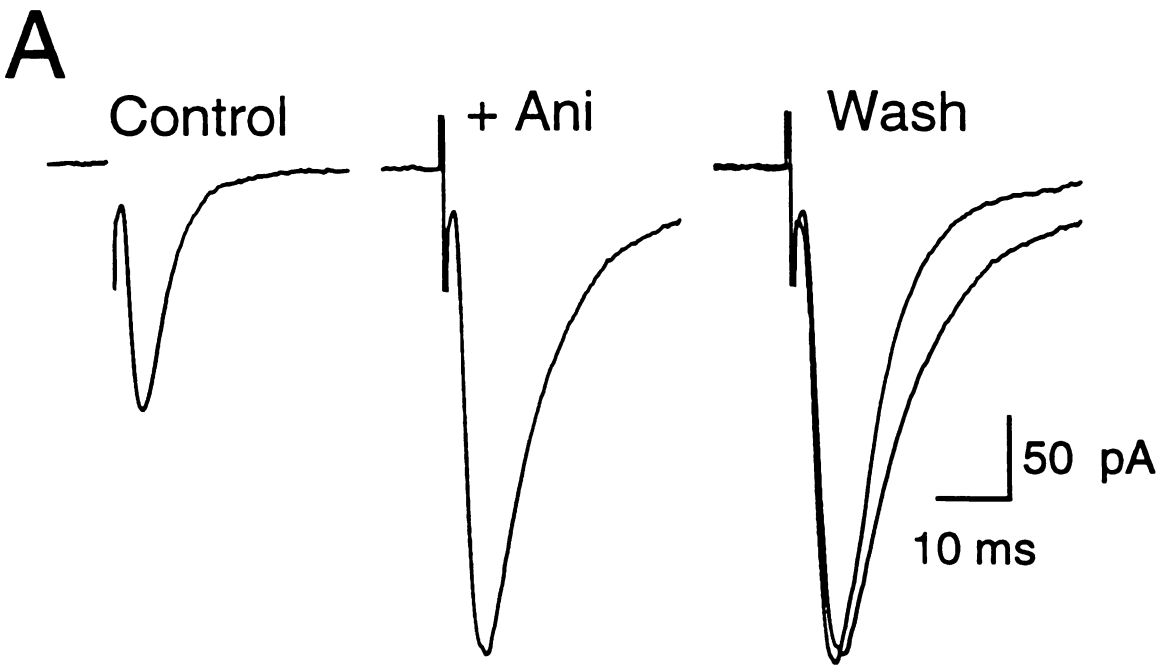

A
Control
Glutamate
Aniracetam
Glutamate
50 pA
200 ms
B
Kainate
Con
+ Ani
5 pA
50 ms

Fig. 8 Aniracetam increases the amplitude and prolongs the time course of non-NMDA receptor-mediated EPSCs. A, Voltage clamp recordings of EPSCs in a granule cell before (Control) and during (+ Ani) the addition of aniracetam (2 mM) to the superfusing medium. After washout of the drug, the stimulus strength was increased to match the peak amplitude of the EPSC in the presence of aniracetam. These responses are shown superimposed (Wash) and emphasize that aniracetam slows the time course of the EPSC. B, A semi-logarithmic plot of the decay time courses of EPSCs recorded in this cell before (C), during (A), and after (W) treatment with the drug. The synaptic currents were well-fitted by single exponential functions with time constants of 4.5 (C), 10.3 (A), and 5.3 (W) ms.


A
Control
+ Ani
Wash
50 pA
10 ms

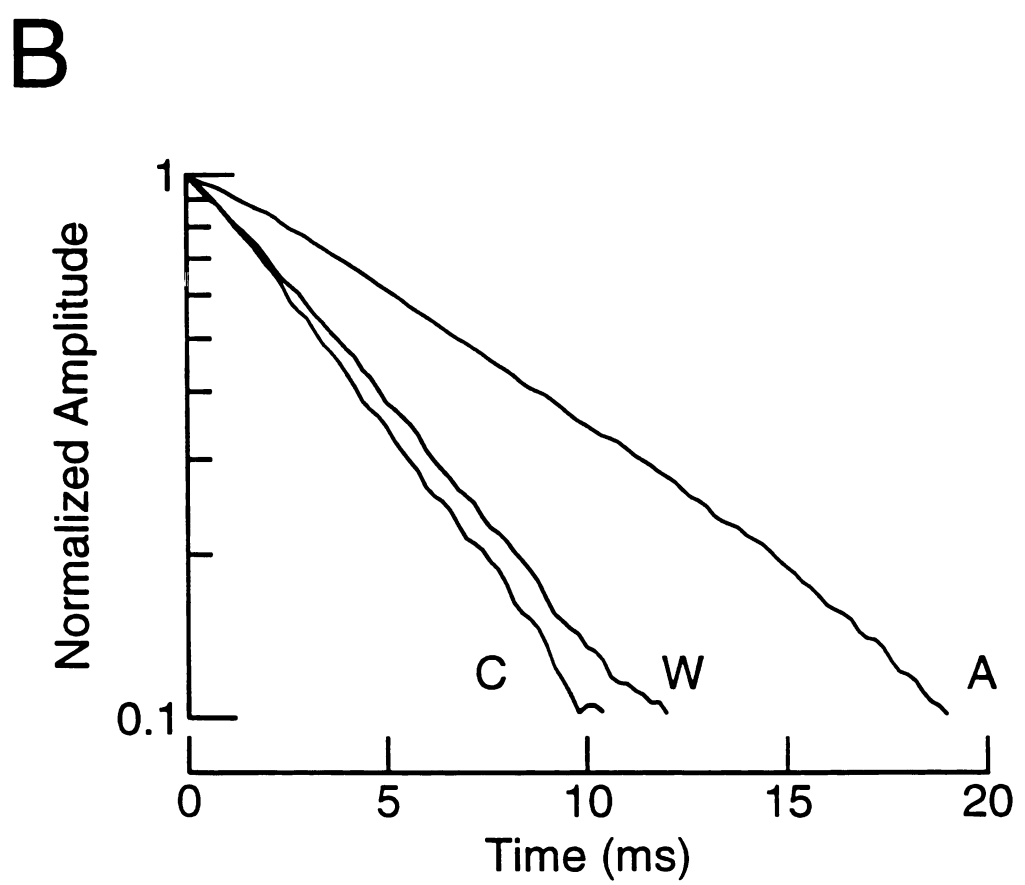

B
Normalized Amplitude
1
0.1
C
W
A
0
5
10
15
20
Time (ms)

Fig. 9 Aniracetam augments the field EPSP and population spike. Field EPSPs and population spikes were recorded simultaneously from two extracellular electrodes placed in the dendritic region and cell layer of CA1, respectively. *Left*, aniracetam (2 mM) increases the amplitude and prolongs the time course of the extracellularly-recorded field EPSP, but has little effect on the initial slope of its rising phase. *Right*, these actions on the field EPSP are accompanied by a large increase in the population spike.

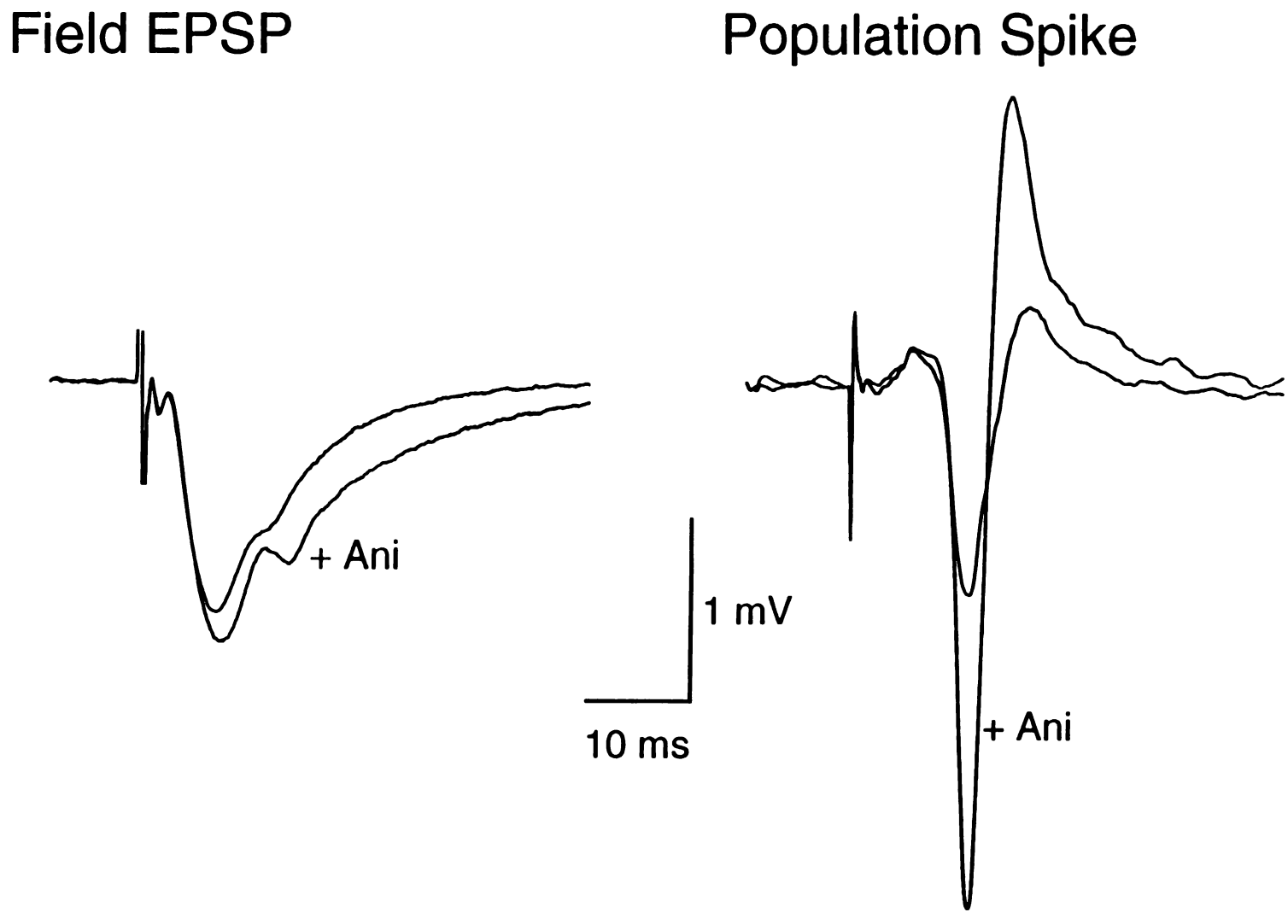

Field EPSP
Population Spike
+ Ani
1 mV
10 ms
+ Ani

Fig. 10 Aniracetam selectively enhances the non-NMDA receptor-mediated EPSC. A, piracetam, a structurally-similar nootropic drug does not effect excititatory synaptic transmission. Synaptic currents in a CA1 cell (Control) were unaffected by the addition of piracetam (3 mM) to the bath (Piracetam), while a subsequent application of aniracetam (+ Ani, 2 mM) caused a large increase in the size of the EPSC. B, aniracetam has no effect on the NMDA receptor-mediated EPSC. B_1, EPSCs from a CA1 cell were first recorded at -80 mV and were increased in size when the superfusing solution was changed to one containing aniracetam (2 mM) (+Ani). After washout of the drug, CNQX (15 μM) was added to the bath and completely blocked the EPSC at this potential (not shown). B_2, the cell was then depolarized to a holding potential of +50 mV and the stimulus strength increased to generate NMDA receptor-mediated EPSCs. Aniracetam had no effect on these synaptic currents and the responses before and after (+Ani) treatment are superimposed. However, the response did increase during the expression of post-tetanic potentiation induced by a 100 Hz/1 s tetanus, as shown by the average of the first two EPSCs immediately following the tetanus (PTP). Synaptic currents were evoked at 0.1 Hz from bipolar stainless steel electrodes placed in s. radiatum for CA1 recordings and the perforant path for recordings from dentate granule cells.

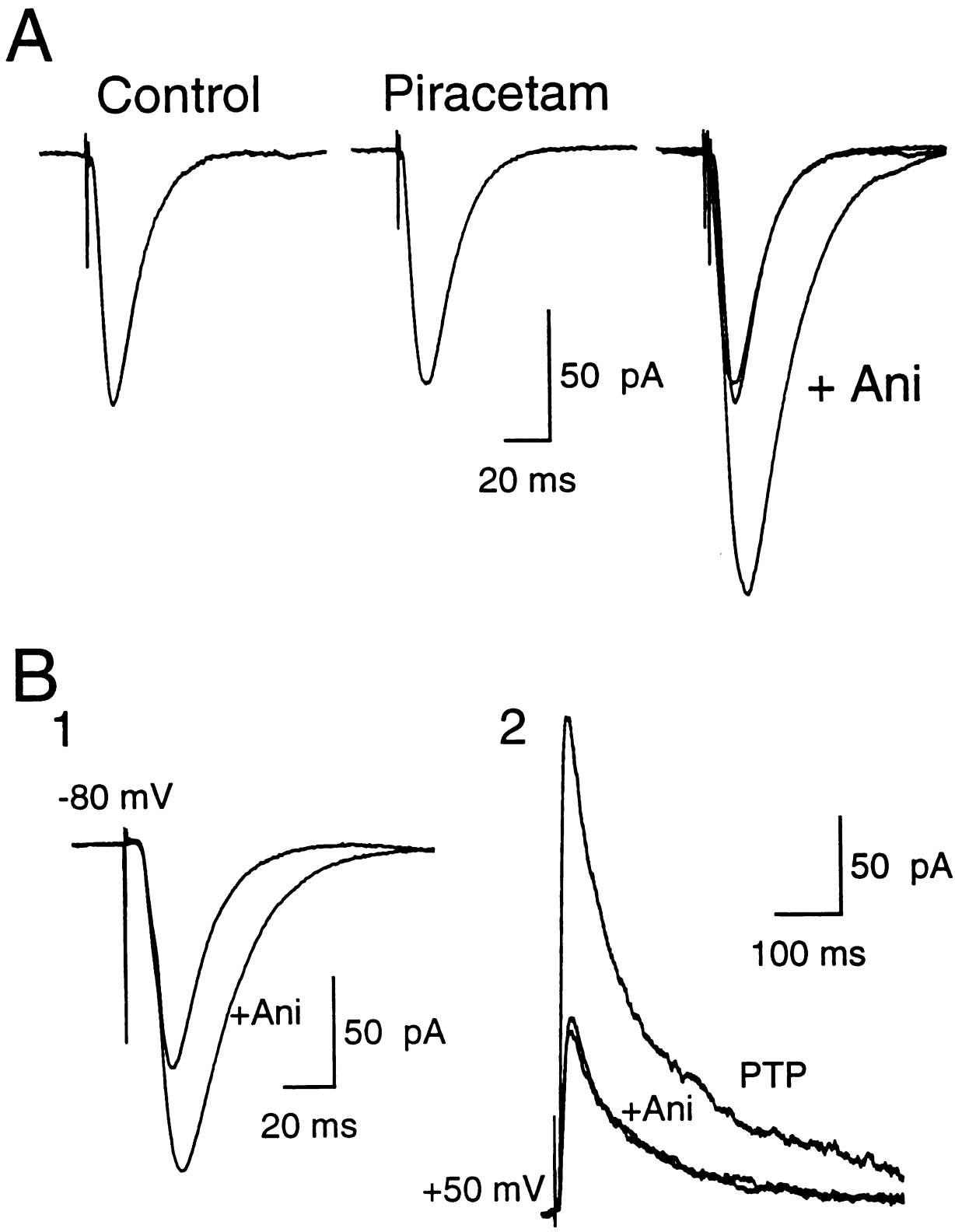

A
Control
Piracetam
50 pA
20 ms
+ Ani
B
1
-80 mV
+Ani
50 pA
20 ms
2
50 pA
100 ms
PTP
+Ani
+50 mV

Fig. 11 Miniature EPSCs (mEPSCs) can be detected in the hippocampal slice. A, miniature synaptic currents recorded from a granule cell (Vh=-80 mV) in the presence of TTX (0.5 μM). Five traces are superimposed. B, histograms of the amplitudes (left) and decay time constants (right) of 500 mEPSCs from the cell illustrated in A.

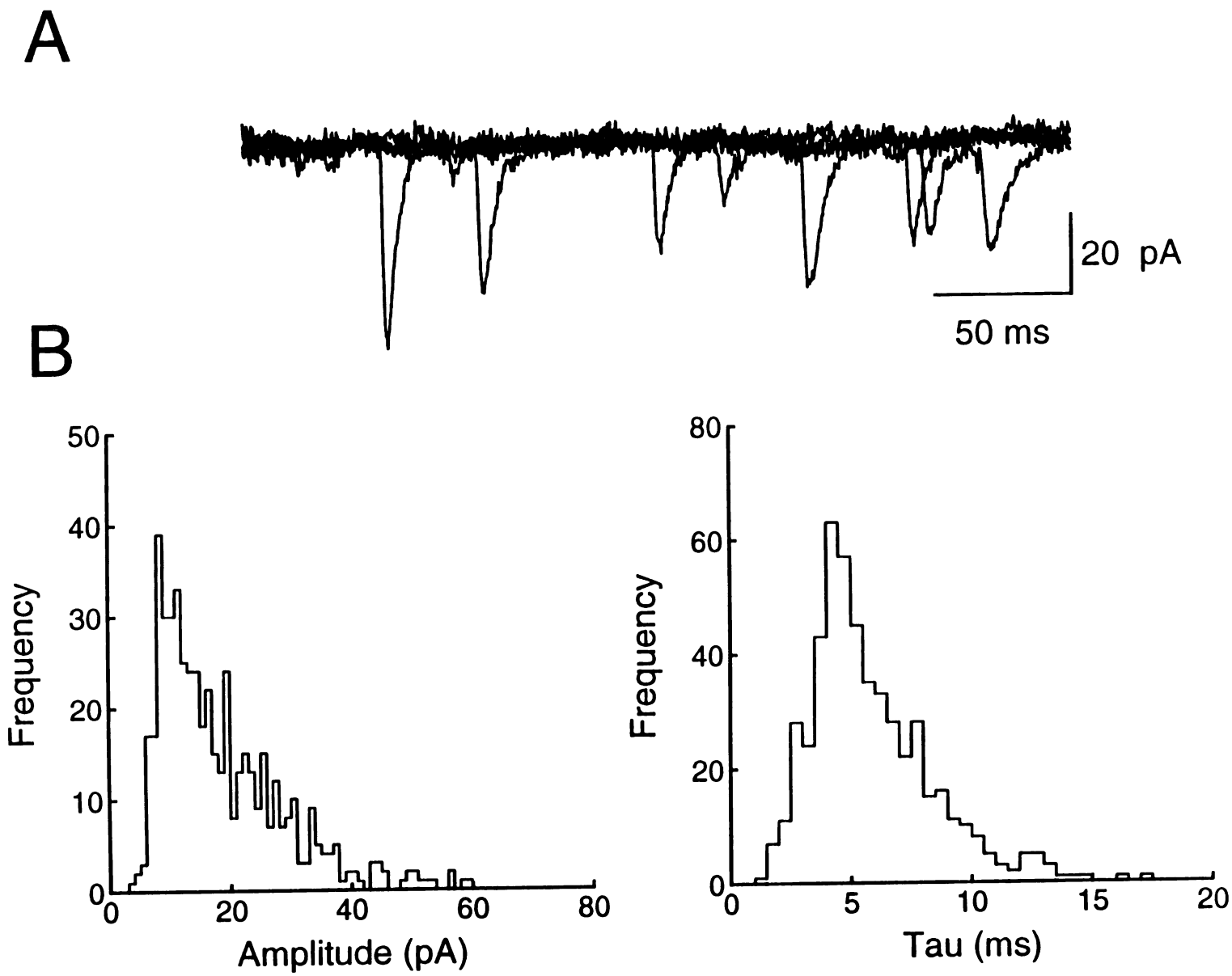

A
20 pA
50 ms
B
Frequency
Amplitude (pA)
Frequency
Tau (ms)

Fig. 12 Aniracetam prolongs the time course and increases the amplitude of miniature EPSCs (mEPSCs). A, shows averages of 50 miniature EPSCs from a granule cell before (Control), during the superfusion of 3 mM aniracetam (Aniracetam) and the superimposition of these records. B_1, chart records of mEPSCs in a CA1 neuron held at -80 mV. Aniracetam (2 mM) caused an increase in the amplitude of mEPSCs in this cell, which reversed upon washout of the drug. B_2, twenty mEPSCs recorded from this cell before, during, and after treatment with aniracetam were aligned and averaged.

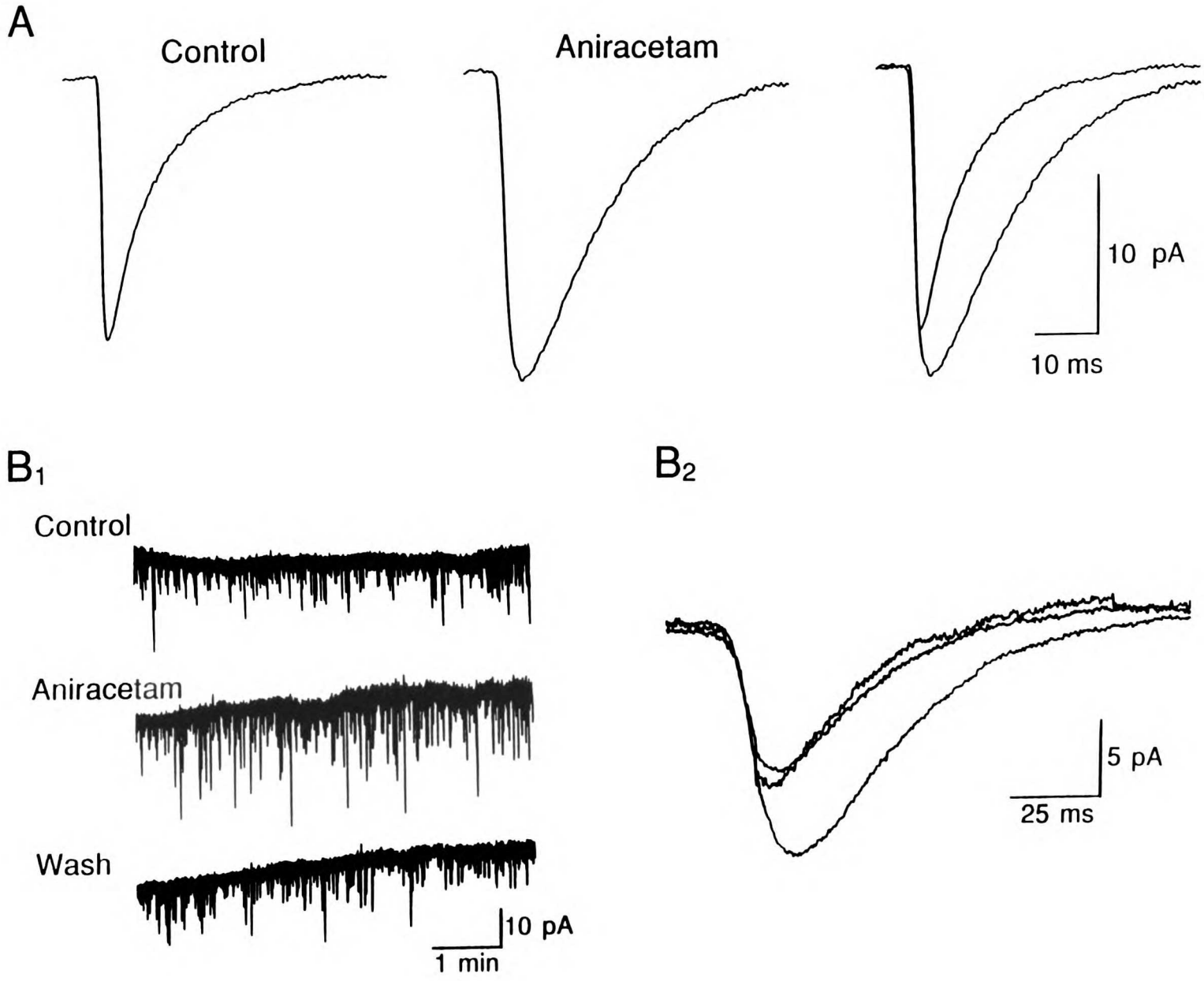

A
Control
Aniracetam
10 pA
10 ms
B1
Control
Aniracetam
Wash
10 pA
1 min
B2
5 pA
25 ms

Fig. 13 Aniracetam reduces the desensitization recorded with paired pulses of quisqualate. A, Paired pulses of quisqualate (100 μM, 10 ms) were delivered at 100 ms intervals. Under normal conditions (Control) the peak response to a second pulse of quisqualate was desensitized by 70% at an interval of 100 ms. The response to the second pulse of quisqualate gradually increased as the receptors recovered from desensitization and paired pulses at intervals of 100 - 500 ms are shown superimposed. Aniracetam (2 mM) was then added to the superfusion solution and the series of paired pulses was repeated (+ Ani). In the presence of aniracetam, the time course of the individual responses to quisqualate were prolonged and the desensitization of the amplitude of the second pulse was greatly reduced. B, the desensitization of the second pulse is plotted (after correction for the decaying current of the first pulse) as the percent of the maximal response. Aniracetam (filled circles) uniformly reduced the amount of desensitization at all intervals (open circles = Control).

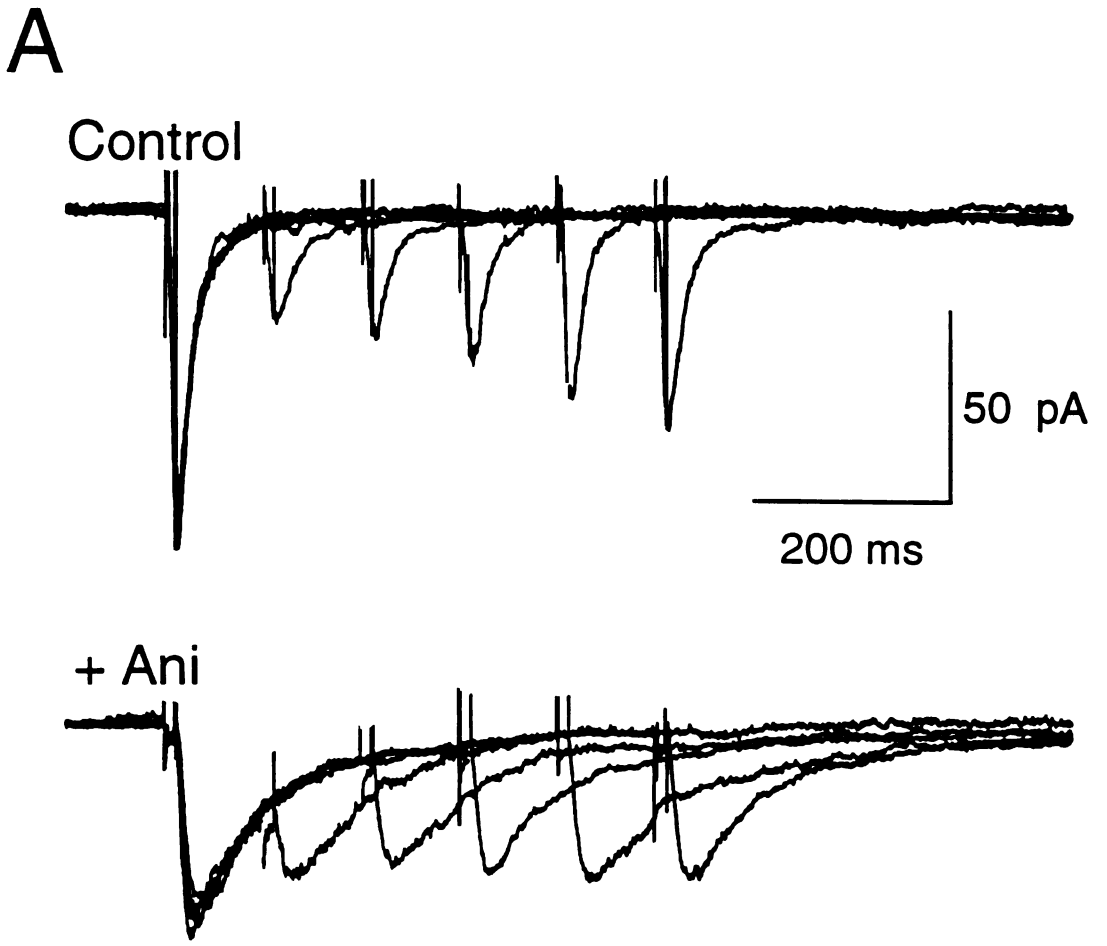

A
Control
50 pA
200 ms
+ Ani
B
% Max Response
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
Interval (mS)

Fig. 14 Aniracetam prolongs responses to brief applications of glutamate. A, responses in the same granule cell patch to glutamate (2 mM) applied for 5 or 100 ms. The desensitization of the response to the long pulse is fit by an exponential curve with a tau of 12.4 ms. The decay of the response to a brief pulse of glutamate is much faster than the time course of desensitization. In this experiment, the response to a brief glutamate pulse is fit by an exponential with a time constant of decay of 3.6 ms. B, the response to very brief applications of glutamate is prolonged by aniracetam. In a patch from a CA1 cell, the time constant of decay of the response to a 6 ms pulse of 2 mM glutamate is 3.8 ms. In the presence of aniracetam (+ Ani) the response is fit with a tau value of 7.9 ms.

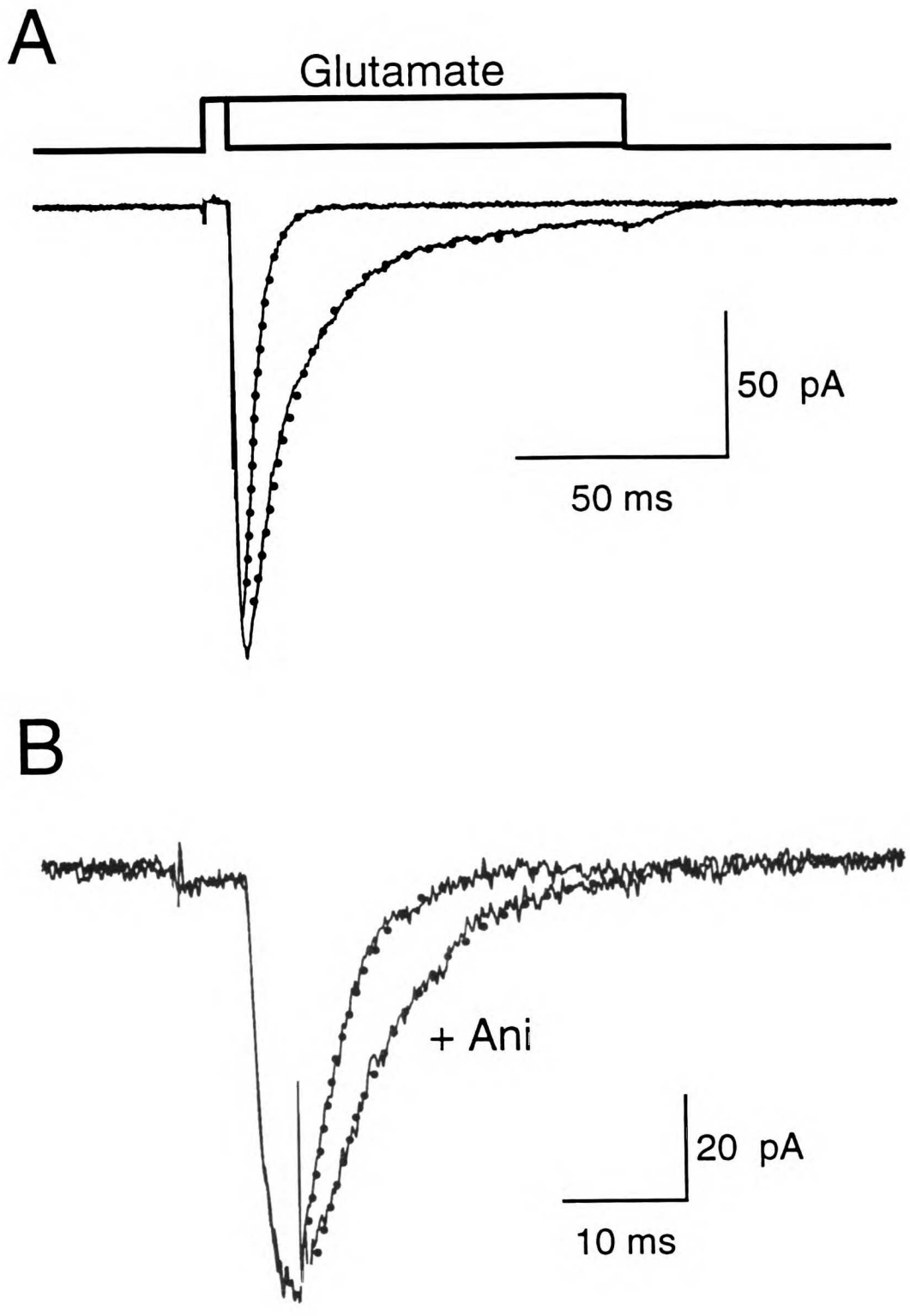

A
Glutamate
50 pA
50 ms
B
+ Ani
20 pA
10 ms

Fig. 15 A comparison of the effect of LTP and aniracetam on the decay of the EPSC. A shows the superimposition of the control response onto the response elicited 10 min after pairing 20 EPSCs at a holding potential of -10 mV. The EPSP showed an approximate 3-fold potentiation (the lower gain refers to the LTP trace). B shows the superimposition of the control response with the response obtained after LTP and after the stimulus had been turned down (Calib. = 50 pA). C shows the superimposition of the control response with the response elicited in the presence of aniracetam. The response in the presence of aniracetam is scaled down to the amplitude of the control response.

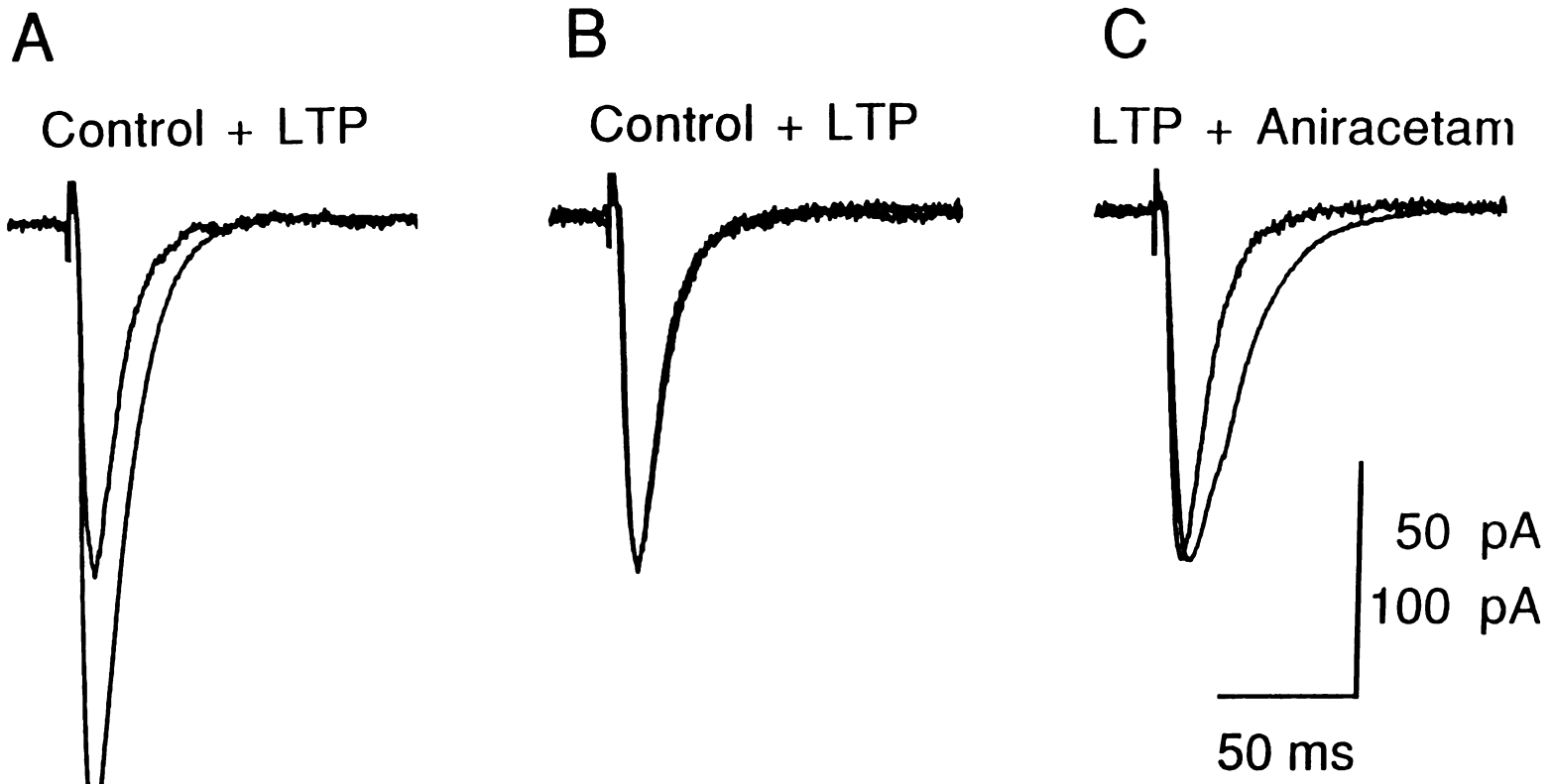
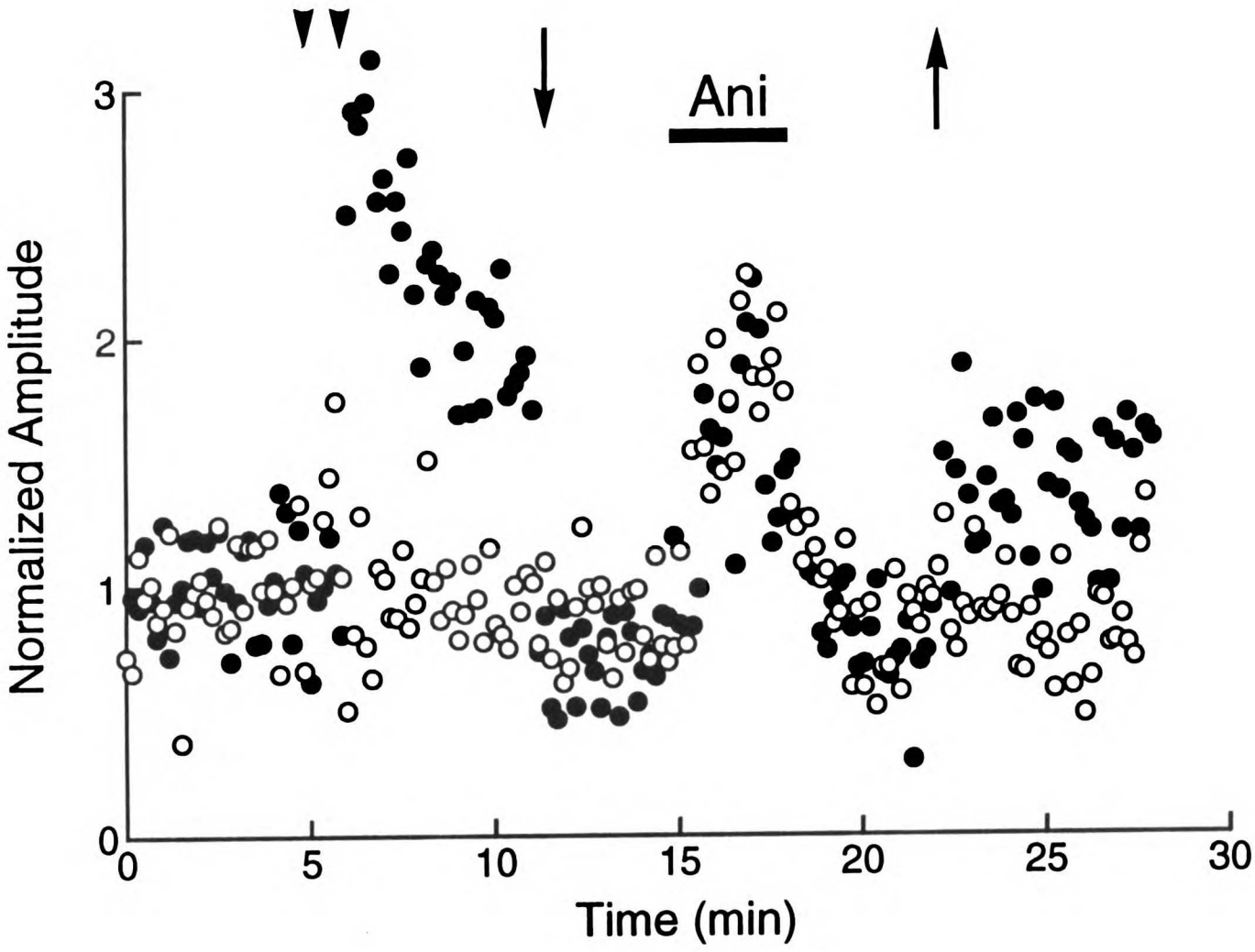

A
Control + LTP
B
Control + LTP
C
LTP + Aniracetam
50 pA
100 pA
50 ms

Fig. 16 The effect of aniracetam on the amplitude of the EPSC does not interact with the enhancement of synaptic strength expressed during LTP. In this experiment, at the time of the double arrow a tetanus (100 Hz, 1 sec repeated twice) was delivered to one of two independent pathways while the cell was held at -10 mV. The stimulus strength of the potentiated pathway (filled circles) was then reduced to match the amplitude of the independent control pathway (see downward arrow). Aniracetam (2 mM) enhanced both sets of inputs equally. After the response to aniracetam had recovered, the stimulus to the tetanized pathway was returned to its original level (see upward arrow).



The influence of GABA-uptake on the time course of fast and slow inhibitory synaptic transmission

I. Postsynaptic inhibition

Introduction

For most neurotransmitters inactivating mechanisms exist which either degrade the transmitter enzymatically or remove the transmitter by reuptake. The effect of transmitter inactivation on the time course of synaptic events recorded at different sites varies considerably. Studies on the role of transmitter inactivation in fast synaptic transmission have focused primarily on nicotinic cholinergic transmission. Here blockade of AChE both at the neuromuscular junction (Katz & Miledi, 1973; Magleby & Terrar, 1975) and at ganglionic cholinergic synapses (Bornstein, 1974) has little effect on the peak amplitude and causes a modest prolongation of synaptic responses (approximately 3 fold). In the absence of AChE, ACh is cleared from the synaptic cleft of the neuromuscular junction by buffered diffusion (Katz & Miledi, 1973; Magleby & Terrar, 1975). Blockage of transmitter removal mechanisms, in general, has a much more dramatic effect on slow synaptic potentials, increasing the peak amplitude and causing a many fold prolongation. Examples include slow muscarinic inhibition of cardiac muscle (Glitsch & Pott, 1978) and ganglion cells (Hartzell, Kuffler, Stickgold & Yoshikami, 1977) by vagal stimulation, slow adrenergic inhibition of submucous and locus coeruleus neurones (Suprenant & Williams, 1987) and slow serotonergic inhibition (Bobker & Williams, 1991).

A number of studies have reported effects of GABA uptake blockade on GABA-mediated synaptic potentials in the CNS (Deisz & Prince, 1989; Dingledine & Korn, 1985; Hablitz & Lebeda, 1985; Rekling et al., 1990).

However, these studies are limited for a number of reasons. Most of the studies used nipecotic acid which is a relatively weak blocker and is itself a substrate at GABA uptake sites (Bernath & Zigmond, 1988; Johnston, Stephanson & Twitchin, 1976; Szerb, 1982). As a result nipecotic acid releases GABA via heteroexchange and substantially elevates the extracellular concentration of GABA which can then activate both postsynaptic receptors as well as autoreceptors. In addition, synaptically released GABA activates both fast $GABA_A$ receptors as well as slow $GABA_B$ receptors. In previous studies it has been difficult to entirely separate the effects of the uptake blocker on these two types of synaptic events.

We have used SKF 89976A, a N-(4,4-diphenyl-3-butenyl) derivative of nipecotic acid (Falch, Larsson, Schousboe & Krogsgaard-Larsen, 1990; Larsson, Falch, Krogsgaard-Larsen & Schousboe, 1988), which is a potent competitive blocker of GABA uptake and is not itself a substrate for the transporter. In this study, we have examined the effects of this blocker on pharmacologically isolated monosynaptic $GABA_A$- and $GABA_B$-mediated synaptic events to determine the influence of transmitter uptake on the time course of postsynaptic inhibition in the hippocampus.

Results

In the presence of glutamate receptor antagonists, stimulation in the dendritic region of CA1 close (within 200 μm) to a recorded pyramidal cell evokes a complex inhibitory current with both fast and slow components (Fig 1A). Since all excitatory transmission in the slice is eliminated, these monosynaptic responses are evoked from the direct stimulation of inhibitory interneurons (Collingridge, Davies & Davies, 1988; Davies, Davies & Collingridge, 1990; Doze, Cohen & Madison, 1991). The fast component of this inihibitory current is mediated by $GABA_A$ receptors since addition of the $GABA_A$-receptor antagonists picrotoxin and bicuculline methiodide (BMI) abolish the response (Fig. 1B). Following the addition of $GABA_A$ antagonists, the remaining slowly rising and decaying current is $GABA_B$ receptor-mediated since this response is completely blocked by the selective $GABA_B$ receptor antagonist, CGP 35348 (Fig. 1C).

In a different fashion, the placement of the stimulating electrode also allows some degree of control of the postsynaptic response. Stimulation close to the soma of CA1 pyramidal cells elicits a relatively pure $GABA_A$ -mediated IPSC, while stimulation farther away from the soma, towards s. lacunosum, evokes $GABA_B$-mediated IPSCs. As discussed previously (see General Introduction), this difference presumably arises from the activation of two classes of inputs, feedback interneurons synapsing on the soma and feedforward interneurons forming dendritic synapses, respectively. Thus, with the appropriate antagonists and electrode placement it is possible to study anatomically- and pharmacologically-isolated $GABA_A$ and $GABA_B$-mediated responses in the slice.

Initial experiments were directed at characterizing the action of SKF 89976A on responses from CA1 hippocampal pyramidal cells. We first

compared the action of SKF 89976A on responses to the somatic application of GABA and THIP, an analogue of GABA which is not a substrate for the GABA transporter (Schousboe, Larsson & Krogsgaard-Larsen, 1985). Bath application of SKF 89976A greatly enhanced the GABA response but had no effect on the response to THIP (n = 5) (Fig. 2). This finding indicates that SKF 89976A can potentiate GABA responses and that the effect is likely to be mediated by the effect on the GABA transporter. To further define the action of SKF 89976A, the response to GABA in outside-out patches pulled from the soma of CA1 neurons was examined. SKF 89976A had no effect on GABA responses recorded in outside-out patches which had been removed from the slice (n = 4) (Fig. 2B). In contrast pentobarbital, which is known to alter the channel kinetics of the GABA receptor (Macdonald, Rogers & Twyman, 1989), prolonged GABA responses in outside-out patches (Fig. 2B). In other experiments with outside-out patches, we found that we could successfully place the patches on the surface of the hippocampal slice (Fig. 3A). A GABA-containing iontophoretic electrode was then placed in the slice, 100-300 μm from the patch electrode on the slice surface. In this configuration, it was possible to evoke GABA-responses in outside-out patches that were enhanced by blocking uptake with SKF 89976A. As shown in Fig. 3B & C, GABA responses in a patch close to the slice were greatly enhanced by SKF 89976A. The simplest explanation for this result is that GABA traversing the distance between the iontophoretic electrode and patch is subject to removal by the GABA transporter. Blocking uptake enhances the responses in these patches by allowing a higher effective concentration of GABA to reach the receptors. These results strongly support the proposal that the enhancement of GABA responses by SKF 89976A results from the inhibition of the removal of GABA by the GABA transporter.

We next examined the effect of blocking GABA uptake on monosynaptic inhibitory currents recorded with whole-cell techniques. Stimulation evoked a fast IPSC followed by a slow IPSC (Fig. 4). SKF 89976A had no effect on the peak of the fast IPSC but appeared to increase the peak and prolong the decay of the slow IPSC. These experiments in which fast and slow IPSCs were evoked together were difficult to interpret since SKF 89976A could be selectively affecting either current or both. We therefore pharmacologically isolated the fast and slow IPSCs.

Fig. 5 shows pure $GABA_B$ receptor mediated IPSCs recorded in the presence of BMI and picrotoxin. Under these conditions, SKF 89976A increased the peak amplitude of the IPSC by 66 ± 9% and prolonged its half decay time by 116 ± 6% (n = 7). This enhancement of the slow IPSC is mediated by $GABA_B$ receptors since the subsequent addition of the antagonist CGP 35348 completely blocks the response (Fig. 5). Another consistent action of SKF 89976A on the slow IPSC was an increase in the time to peak of the response. As shown in Fig. 6, while an increase in the stimulus strength was accompanied by an increase in the rate of rise of the slow IPSC, the enhancement caused by SKF 89976A had no effect on the rising phase of the synaptic current.

We next studied the effect of blocking GABA uptake on $GABA_A$ receptor mediated IPSCs recorded in the presence of the $GABA_B$ antagonist CGP 35348 (Fig. 7). SKF 89976A had no effect on the peak of the IPSC or the initial decay. However, it greatly prolonged the remaining third of the IPSC which required more than a second to completely decay. Bicuculline methiodide (BMI) was added to the potentiated response (see superimposed recorded in + SKF 89976A) to verify that the entire response was mediated by $GABA_A$ receptors.

Based on models of fast synaptic transmission (Korn & Faber, 1987; Wathey, Nass & Lester, 1979) including GABA synapses in the hippocampus (Busch & Sakmann, 1990) it is postulated that transmitter diffuses from the cleft within a few milliseconds and the decay of the synaptic current reflects the lifetime of the open channel. If this is the case, the observed prolongation of the fast IPSC might result from the simultaneous release of GABA from many synapses and the spread of GABA to neighboring synapses. To test this proposal we compared the effect of SKF 89976A on weak and strong stimulation. Under normal conditions, IPSCs evoked with a weak stimulus had a time course quite similar to those evoked in the same cell with a stronger stimulus (Fig. 8). The inhibition of GABA-uptake by SKF 89976A had little effect on the IPSC evoked with a weak stimulus. Similar results were obtained with spontaneous IPSCs (Fig. 9) which represent both action potential dependent and independent events. These findings are in agreement with models of fast synaptic transmission in which the diffusion of GABA from the synaptic cleft is fast relative to the time course of the synaptic current. When a strong stimulus was used, however, SKF 89976A greatly prolonged the late phase of the IPSC which now required more than a second to decay completely, but had no effect on the amplitude or initial decay of the IPSC (Figs. 8 & 9). The selective action of SKF 89976A on IPSCs evoked with strong versus weak stimuli was found in ten slices. These results suggest that when many neighboring synapses are activated simultaneously, during a strong stimulus, and uptake is blocked, GABA "spills-over" from adjacent synapses and can pool to reach concentrations high enough to activate postsynaptic receptors.

The effect of SKF 89976A on the spill over of GABA was directly examined using outside-out patches placed near the surface of the slice as

sensors of GABA concentration. Surprisingly, in control conditions paired pulse stimulation using high stimulus strengths often evokes brief channel activity in outside-out patches (Fig. 10). In the presence of SKF 89976A many more channels were activated and the activity was greatly prolonged. Withdrawal of the patch from the surface of the slice (Fig. 10) or addition of bicuculline methiodide (not shown) abolished the channel activity. Similar results were found using three other patches in different slices.

In the presence of SKF 89976A, $GABA_A$-mediated IPSCs evoked with strong stimulation could last for longer than a second. This long lasting effect of GABA is longer than one might expect if free diffusion cleared the transmitter from the slice (Eccles & Jaeger, 1958). One possibility is that the diffusion of transmitter, released from neighboring synapses, is "buffered" by repeated binding to postsynaptic receptors (Katz & Miledi, 1973). This buffered diffusion accounts for the prolonged time course of the endplate potential at the NMJ when acetylcholinesterase is blocked, since treatment of the endplate with acetylcholine receptor antagonists, which prevent transmitter binding, increases the rate of decay of the endplate currents (Katz & Miledi, 1973; Magleby & Terrar, 1975). To address this possibility, we examined the effect of the competitive $GABA_A$ antagonist, BMI, on the time course of the IPSC in the presence of SKF 89976A. Under normal conditions BMI has no effect on the time course of the fast IPSC (not shown), however, in four slices the antagonist markedly increased the rate of decay of the IPSC in the presence of the uptake inhibitor (Fig. 11). Therefore, as is the case at the NMJ when acetylcholinesterase is blocked, these results suggest that buffered diffusion prolongs the lifetime of GABA at postsynaptic receptors when the GABA transporter is inhibited.

Discussion

In this study we have found that the glial and neuronal GABA-uptake blocker SKF 89976A has powerful effects on GABA-mediated responses in the hippocampus. SKF 89976A selectively enhanced responses to exogenously-applied GABA but had no effect on responses to THIP, an analogue of GABA which is not a substrate for the GABA transporter. The lack of effect of SKF 89976A on GABA responses in outside-out patches positioned far from the slice provides further support that the effects of the compound are not direct ones, but rather reflect actions mediated *via* an inhibition of GABA-uptake in the slice.

Effect of SKF 89976A on the $GABA_B$-mediated IPSC

In contrast to the ligand-gated Cl^- channels underlying fast $GABA_A$-mediated inhibition, the slow, $GABA_B$-mediated IPSP has been shown to represent the activation of a potassium conductance via a G-protein coupled receptor (Dutar & Nicoll, 1988b; Newberry & Nicoll, 1985; Thalmann, 1987; Thalmann, 1988). Factors such as the kinetics of G-protein-effector interactions or the continued presence of GABA may contribute to determining the time course of this slow synaptic potential. However, the precise factors controlling the time course of the $GABA_B$-mediated IPSC are unknown and the nature of the synaptic architecture mediating the slow IPSC is still unresolved.

Recently, it has become clear that $GABA_A$- and $GABA_B$-mediated synaptic responses can be segregated at different locations on the same postsynaptic neuron (Otis & Mody, 1992a; Solís & Nicoll, 1992b; Sugita et al., 1992; Williams & Lacaille, 1992). Furthermore, high levels of stimulation or other conditions favoring large amounts of transmitter release are often

required to evoke a slow IPSC (Dutar & Nicoll, 1988b; Otis & Mody, 1992a). One possibility is that the slow IPSC represents the activation of diffusely distributed receptors. In support of this argument, we have found that the blockade of GABA-uptake, which could increase the spatial and temporal domain of transmitter, markedly enhanced the slow IPSC. However, in contrast to the effect of increasing stimulus strength, which allows the recruitment of new receptors, SKF 89976A did not increase the rate of rise of the slow IPSC. The action of SKF 89976A to increase the time to peak of the synaptic response suggests that the rising phase of the slow IPSC is determined by the spread of GABA to postsynaptic receptors. Although generally accepted (but see Muller & Misgeld, 1989), the enhancement of the slow IPSC by the selective GABA-uptake inhibitor also provides additional strong evidence that GABA is, in fact, the neurotransmitter mediating slow inhibitory transmission in the hippocampus (Dutar & Nicoll, 1988a; Newberry & Nicoll, 1985; Solís & Nicoll, 1992a; Soltesz, Haby, Leresche & Crunelli, 1988). However, a clearer description of the synapses mediating this response requires a better understanding of the localization of the $GABA_B$ receptors on the postsynaptic cell.

Effect of SKF 89976A on the $GABA_A$-mediated IPSC

Our results are in general agreement with a number of studies (Dingledine & Korn, 1985; Rekling et al., 1990; Thompson & Gähwiler, 1992) that have found that other GABA-uptake inhibitors had little effect on the amplitude or initial decay of IPSPs, but greatly augmented their late decay. This result is seemingly inconsistent with models of fast synaptic transmission which suggest that transmitter diffuses from the cleft within a few milliseconds (Busch & Sakmann, 1990 ; Korn & Faber, 1987; Wathey et al.,

1979). An important difference between the two approaches is that these models of synaptic transmission reconstruct the properties of an isolated, individual synapse while physiological studies in brain slices of evoked synaptic responses usually reflect the properties of many neighboring synapses that are simultaneously active. Indeed SKF 89976A had no effect on either miniature IPSCs or those evoked with weak stimulation, which activates fewer synapses, in contrast to its effects on IPSCs evoked by strong stimuli. A similar discrepancy between the actions of a GABA-uptake inhibitor on evoked versus miniature IPSCs has recently been found in hippocampal slice cultures (Thompson & Gähwiler, 1992). These results suggest that GABA-uptake is important for limiting the spread of GABA released by neighboring, co-activated synapses. In the presence of uptake inhibitors, the spill-over of GABA from co-activated boutons may reach $GABA_A$ receptors at neighboring synapses or those at an extrasynaptic location. The idea that SKF 89976A acts to promote the spill-over of transmitter is also supported by our measurements of evoked transmitter release in the slice with a GABA-sensing outside-out patch. The lateral diffusion of transmitter from one discrete post-synaptic domain to another one nearby has previously been shown to occur when neighboring glycinergic synapses on the Mauthner cell are co-activated (Faber & Korn, 1988). Similarly, at the neuromuscular junction, acetylcholinesterase inhibitors produce a "post-synaptic potentiation" by permitting the spread of transmitter to overlapping synaptic domains (Hartzell, Kuffler & Yoshikami, 1975). Furthermore, as at the NMJ (Katz & Miledi, 1973; Magleby & Terrar, 1975), we have found that the buffered diffusion of transmitter slows the removal of GABA from the slice when GABA-uptake is blocked.

Would neighboring GABAergic terminals be co-activated physiologically? Pyramidal neurons in the CA3 region of the hippocampus have been shown to be highly sensitive to elevations in extracellular K^+ or to the K^+-channel blocker 4-aminopyridine and respond with synchronized burst discharges (Dingledine & Korn, 1985; Perreault & Avoli, 1992) which are relayed to pyramidal cells in CA1 (Perreault & Avoli, 1992). This strong, concerted excitatory input would be expected to result in the concomitant activation of large populations of GABAergic interneurons in CA1 as well as CA3. In addition, the property of interneurons to fire in bursts (Schwartzkroin & Mathers, 1979) would greatly accentuate the concentrations of GABA released from closely opposed terminals. Under these conditions, GABA-uptake inhibition may be found to exert a powerful anti-epileptic effect. Indeed, the uptake inhibitor cis-4-OH-nipecotic acid has been shown to strongly reduce synchronous burst firing in CA3 pyramidal cells generated by an increase in extracellular K^+ (Dingledine and Korn, 1985).

Taken together, these results indicate that transmitter uptake governs the strength of slow postsynaptic inhibition in the hippocampus. GABA-uptake also has a strong influence on fast postsynaptic inhibition. It serves to localize fast $GABA_A$-mediated transmission by preventing its spill-over to neighboring synapses.

Figures

Fig. 1 Monosynaptic dual component IPSC is mediated by $GABA_A$ and $GABA_B$ receptors. IPSCs were recorded in the presence of glutamate receptor antagonists in a voltage-clamped CA1 pyramidal cell (Vh= -60 mV). *A*, under control conditions compound IPSC has both fast and slow components. *B*, bicuculline methiodide (+ BMI, 40 μM) selectively blocks the fast component of the IPSC indicating that $GABA_A$ receptors underly this synaptic current. The remaining slow component is $GABA_B$ receptor-mediated since it is blocked by the subsequent addition of CGP 35348 (+ CGP, 500 μM) to the superfusing solution. C, the trace shown in A and the IPSC in the presence of BMI are shown superimposed.

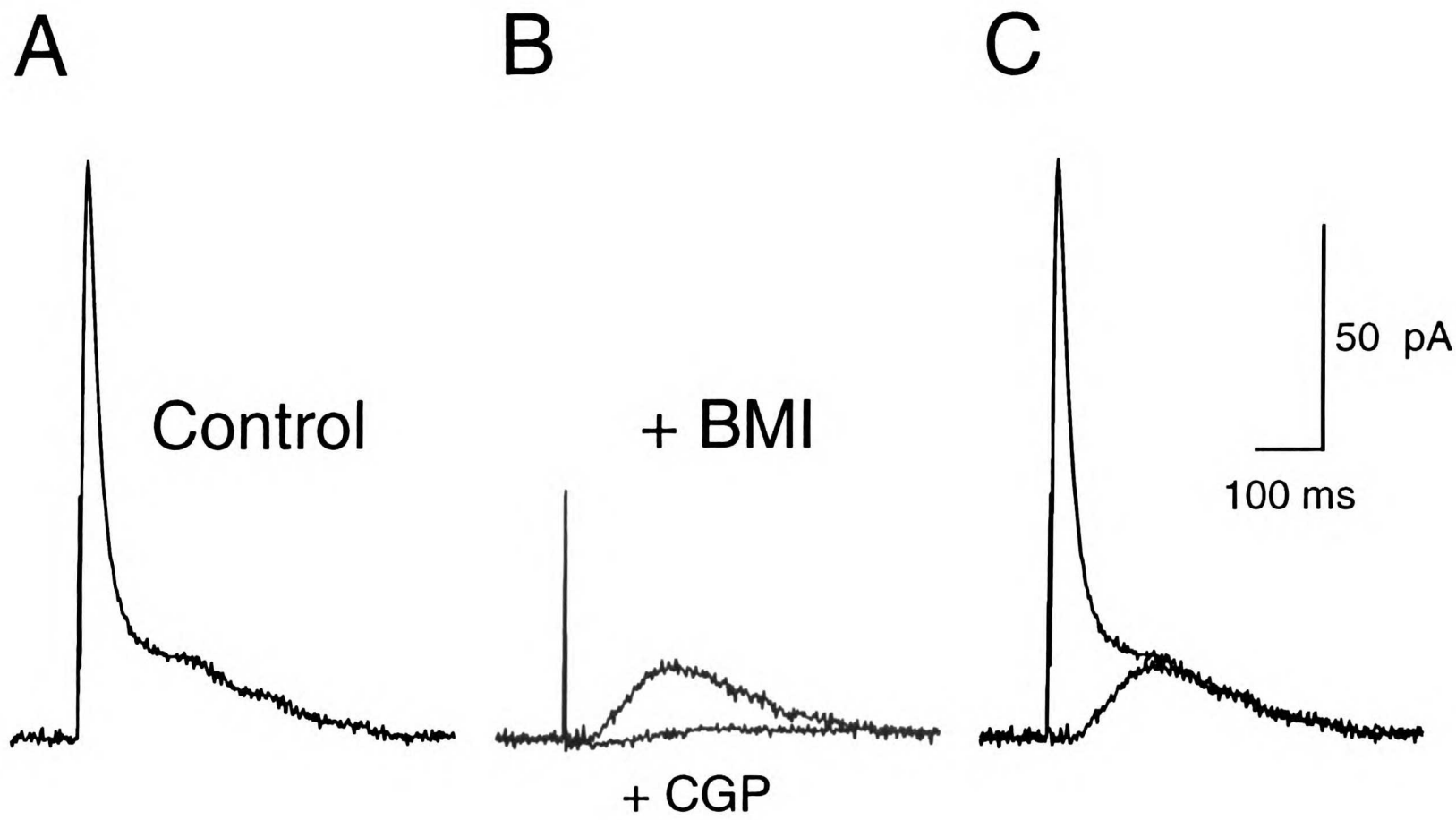

A
B
C
Control
+ BMI
+ CGP
50 pA
100 ms

Fig. 2 The GABA-uptake inhibitor SKF 89976A selectively enhances the responses to GABA applied to cells within the hippocampal slice, but has no direct effect on GABA receptor-channels in outside-out membrane patches. (*A*) Chart record of membrane current from a voltage-clamped CA1 pyramidal cell (V_h = -70 mV). Brief iontophoretic pulses of GABA (▼ , 100nA/1s) and THIP (∇ , 150 nA/ 2s) were alternately applied from separate electrodes positioned close to the cell soma. SKF 89976A (20 uM), added to the superfusing solution during the time indicated by the bar, greatly enhanced the GABA-induced current but had no effect on the responses to THIP. (*B*) Responses to GABA (25 uM) in an outside-out patch taken from a CA1 cell (V_h = -70 mV). GABA was applied via puffer pipette (15 psi/50 ms) at the time indicated (▼) and all records are the average of ten applications. GABA evokes a macroscopic inward current that is unaffected by the addition of SKF 89976A (30 uM) to the superfusing solution. In the same patch, the addition of 100 uM pentobarbital (Pb) to the superfusing solution greatly enhanced the GABA-induced current and this record is shown superimposed over the responses obtained before and after treatment with SKF 89976A.

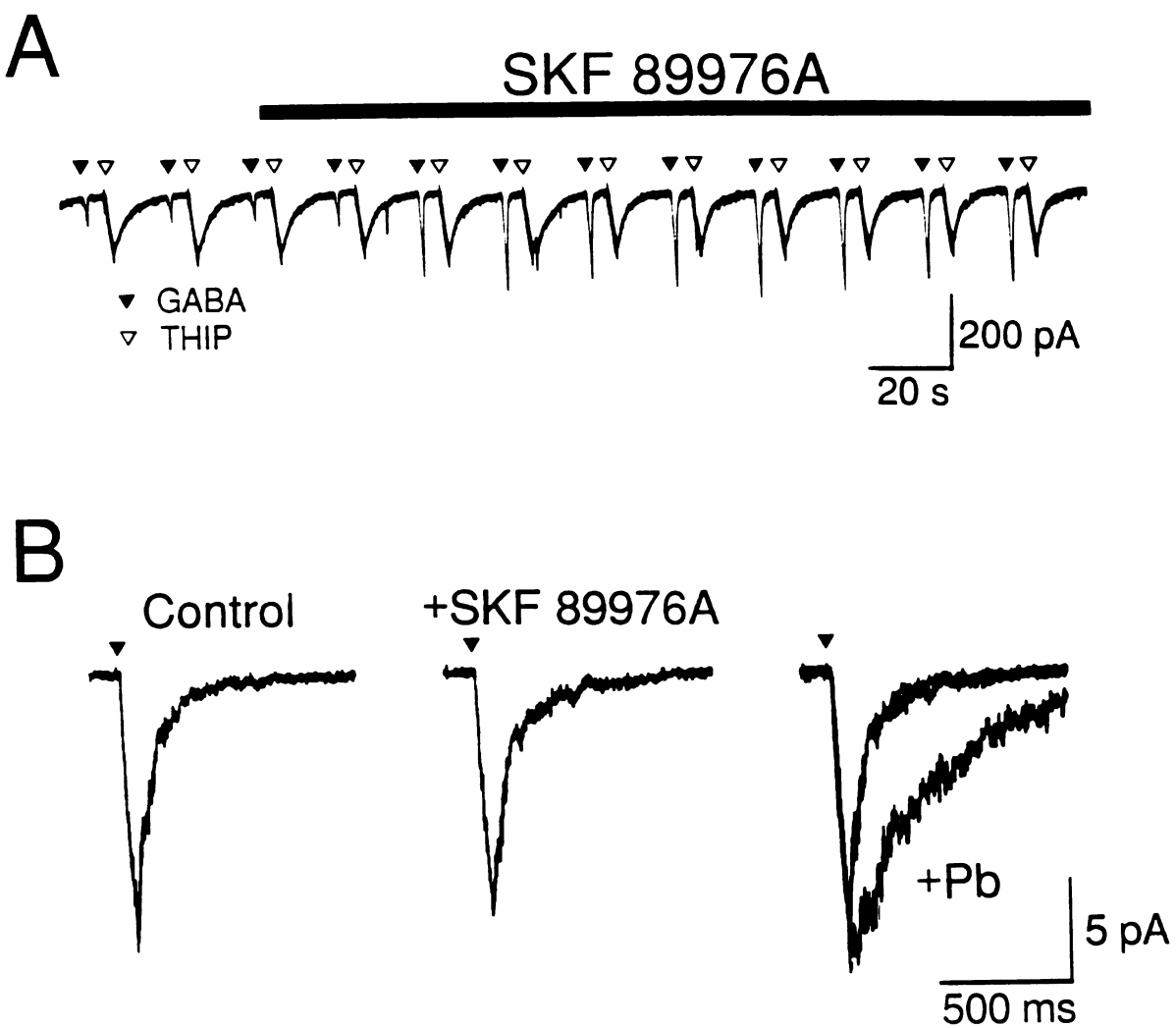

A
SKF 89976A
GABA
THIP
200 pA
20 s
B
Control
+SKF 89976A
+Pb
5 pA
500 ms

Fig. 3 GABA-evoked responses in outside-out patches are enhanced by SKF 89976A when the exogenously-applied transmitter and the patches themselves are in contact with the hippocampal slice. An outside-out patch was pulled from the soma of a pyramidal cells (Vh =-70 mV). As illustrated in *A*, the ouside-out patch was positioned at the surface of the slice, in close proximity to a GABA-filled puffer pipette (25 μM) that was embedded in the slice. *B*, chart record of membrane current from an outside-out patch in this configuration. GABA applied from the puffer pipette (100 ms, 20 psi) every ten seconds evokes a small response in the patch. This response is greatly enhanced following the addition of SKF 89976A (25 μM) to the superfusing solution for the period marked by the bar. *C*, the average of 5 -10 responses recorded before (Control) and after (+ SKF 89976A) the addition of the uptake inhibitor are shown. SKF 89976A both increased the amplitude and prolonged the time course of the GABA responses evoked within the slice andthe two traces are shown superimposed to the right.

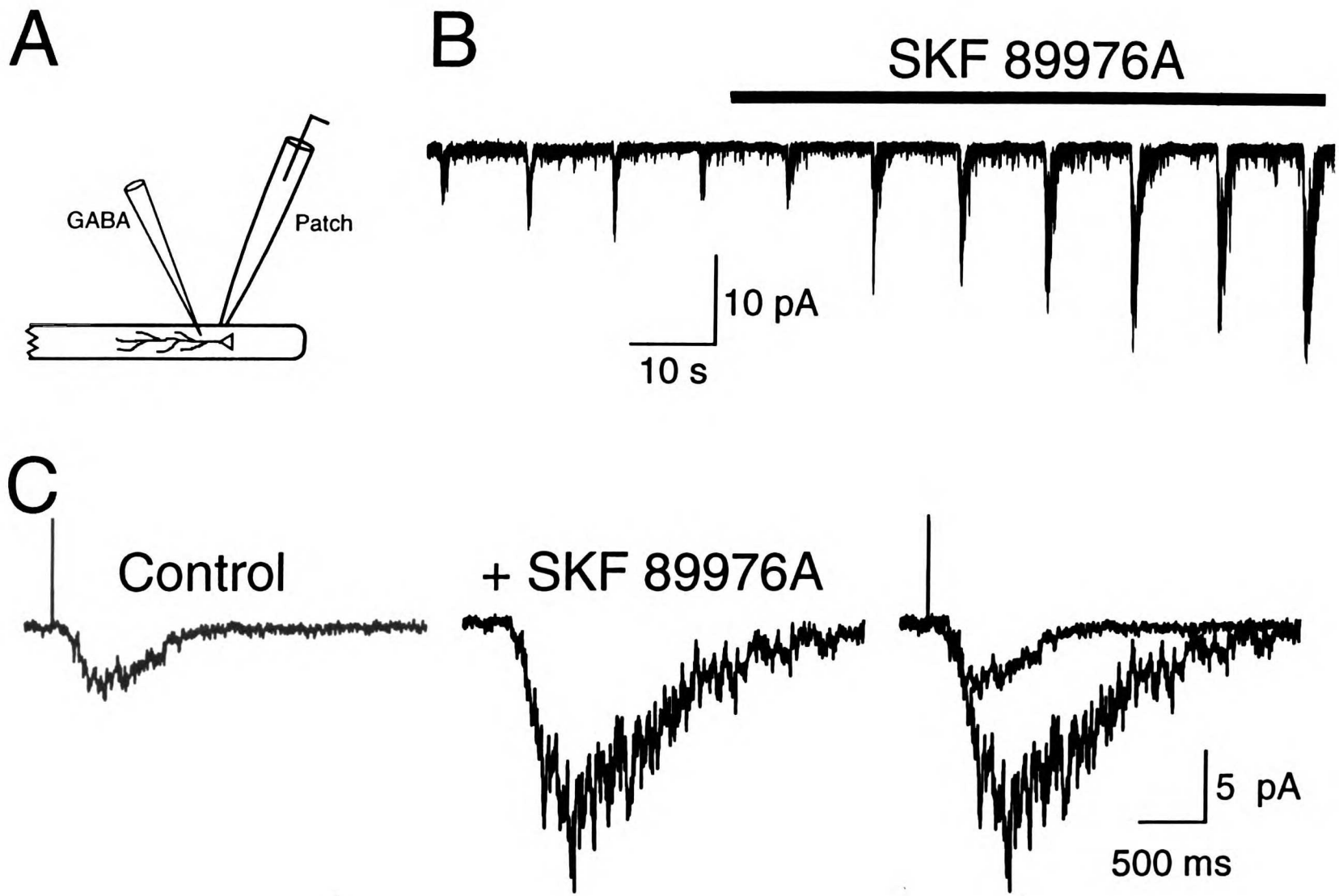

A
GABA
Patch
B
SKF 89976A
10 pA
10 s
C
Control
+ SKF 89976A
5 pA
500 ms

Fig. 4 SKF 89976A enhances monosynaptic inhibitory synaptic currents. Dual component IPSCs recorded from a voltage-clamped granule cell were evoked from a bipolar stimulating electrode placed in the inner molecular layer (Vh = -55 mV). SKF 89976A (20 uM), added to the superfusing media, prolonged the duration of the compound IPSC with little effect on its peak amplitude. The responses before and after treatment with the drug are shown superimposed to the right.

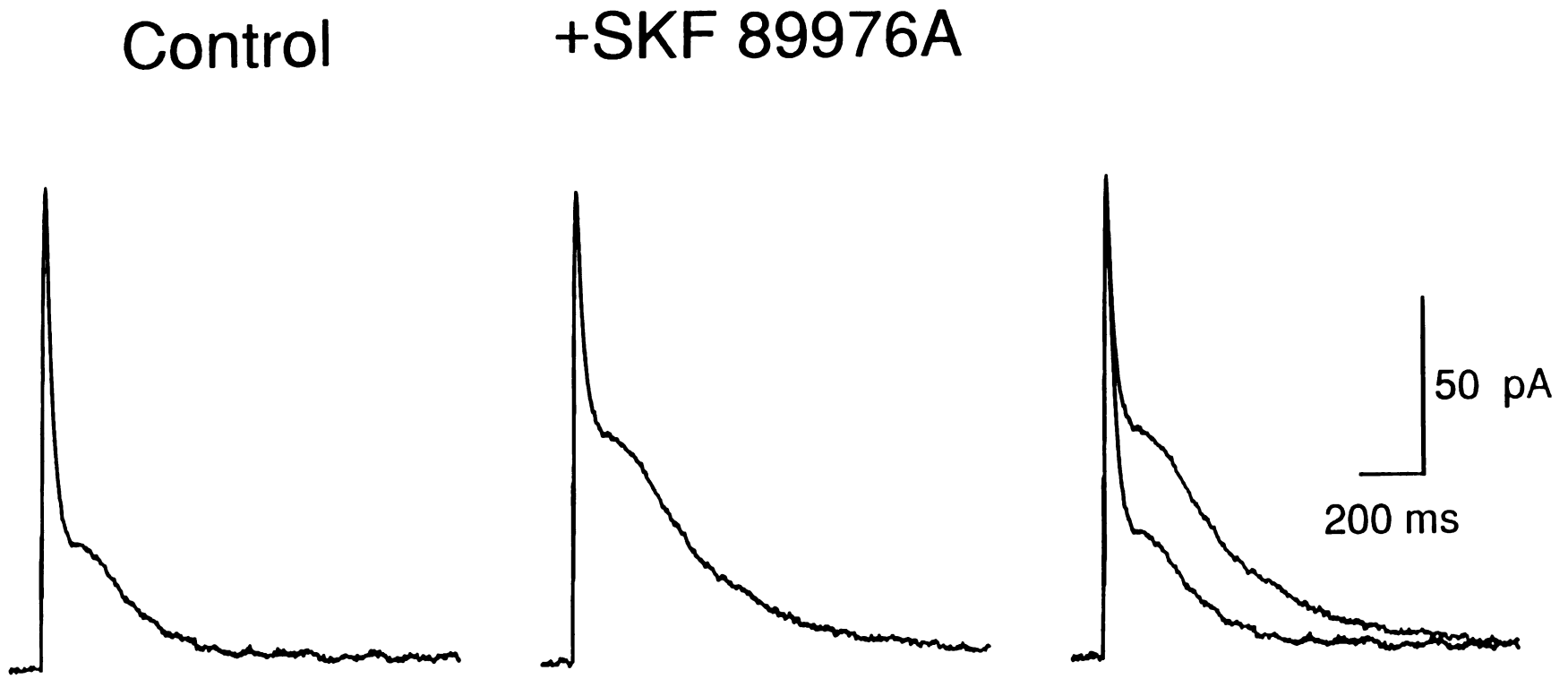

Control
+SKF 89976A
50 pA
200 ms

Fig. 5 The $GABA_B$-receptor mediated component of the monosynaptic IPSC is augmented by SKF 89976A. Monosynaptic $GABA_B$-receptor mediated IPSCs recorded from a voltage-clamped CA1 neuron (K-gluconate internal solution, Vh = -60 mV) in the presence of picrotoxin (50 uM) and BMI (40 uM). *Left*, $GABA_B$ IPSCs were evoked from a stimulating electrode placed in stratum radiatum. *Middle*, SKF 89976A (20 uM) caused a marked increase in both the amplitude and time course of the synaptic current. These responses were blocked following the addition of CGP 35348 (400 uM) to the bath solution. *Right*, the records before and after treatment with SKF 89976A are superimposed.

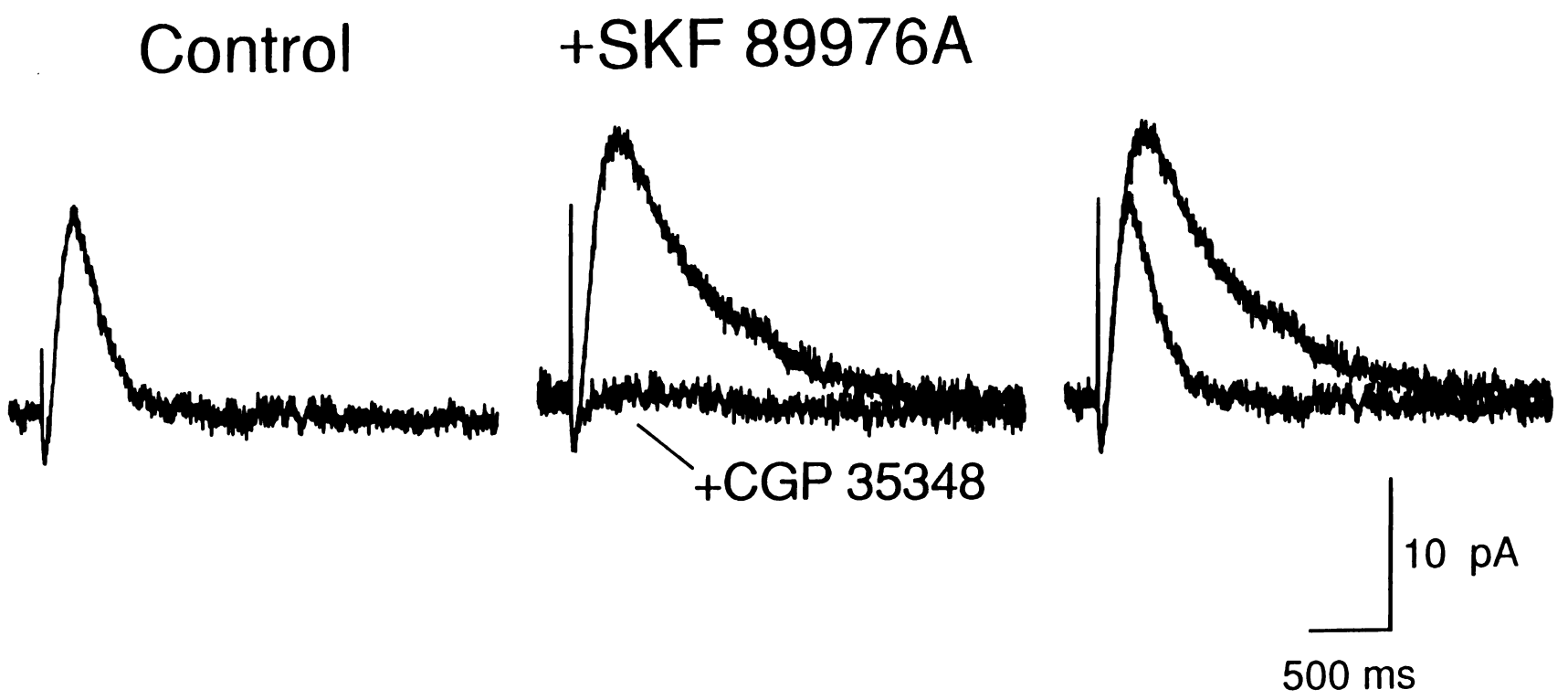

Control
+SKF 89976A
+CGP 35348
10 pA
500 ms

Fig. 6 Comparison between increasing the stimulus strength and SKF 89976A on the rising phase of the $GABA_B$-mediated IPSC. The same recording conditions as Fig. 5 were used. *Top*, slow IPSCs were alternately evoked with weak (W, 10V) and strong (S, 40 V) stimulation and are shown superimposed to the left. Increasing the stimulation strength increases the amplitude of the slow IPSC and is accompanied by an increase in slope of the rising phase of the synaptic current. Thus, as shown to the right, when the traces are scaled so that their amplitudes match the two traces appear identical. SKF 89976A (+ SKF, 25 μM) prolonged the time course and increased the amplitude of the IPSCs evoked with both weak (*middle*) and strong (*bottom*) stimulus strengths. However, the uptake inhibitor increased the time to peak amplitude of the responses. As shown to the right, when the traces are scaled, the IPSCs in the presence of SKF 89976A peak later than the control responses.

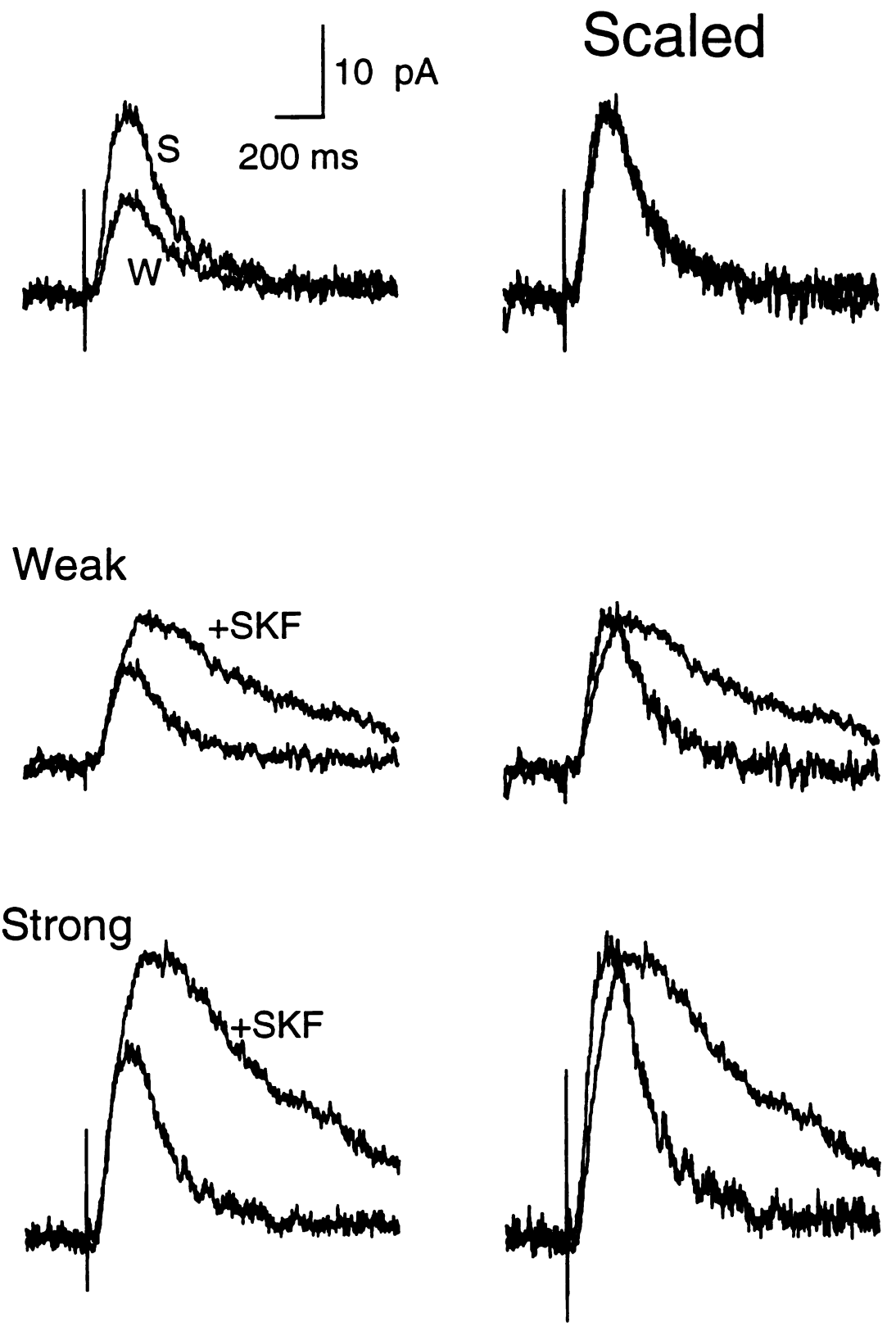

Scaled
10 pA
200 ms
S
W
Weak
+SKF
Strong
+SKF

Fig. 7 SKF 89976A prolongs the time course of the fast IPSC. Monosynaptic $GABA_A$-receptor mediated IPSCs were recorded with a Cs-based internal solution from a patch-clamped CA1 pyramidal neuron (Vh = -55 mV) bathed in media containing the $GABA_B$-receptor antagonist CGP 35348 (400uM). *Left*, synaptic currents were evoked from a bipolar stimulating electrode placed close to the cell in stratum pyramidale. *Middle*, SKF 89976A (25 uM) when added to the superfusing solution, greatly prolonged the time course of the IPSC but had little effect on the peak amplitude and early decaying phase of the synaptic current. The subsequent addition of BMI (40 μM) to the bath completely blocked the synaptic response. *Right*, the traces before and after addition of SKF 89976A to the bath are shown superimposed.

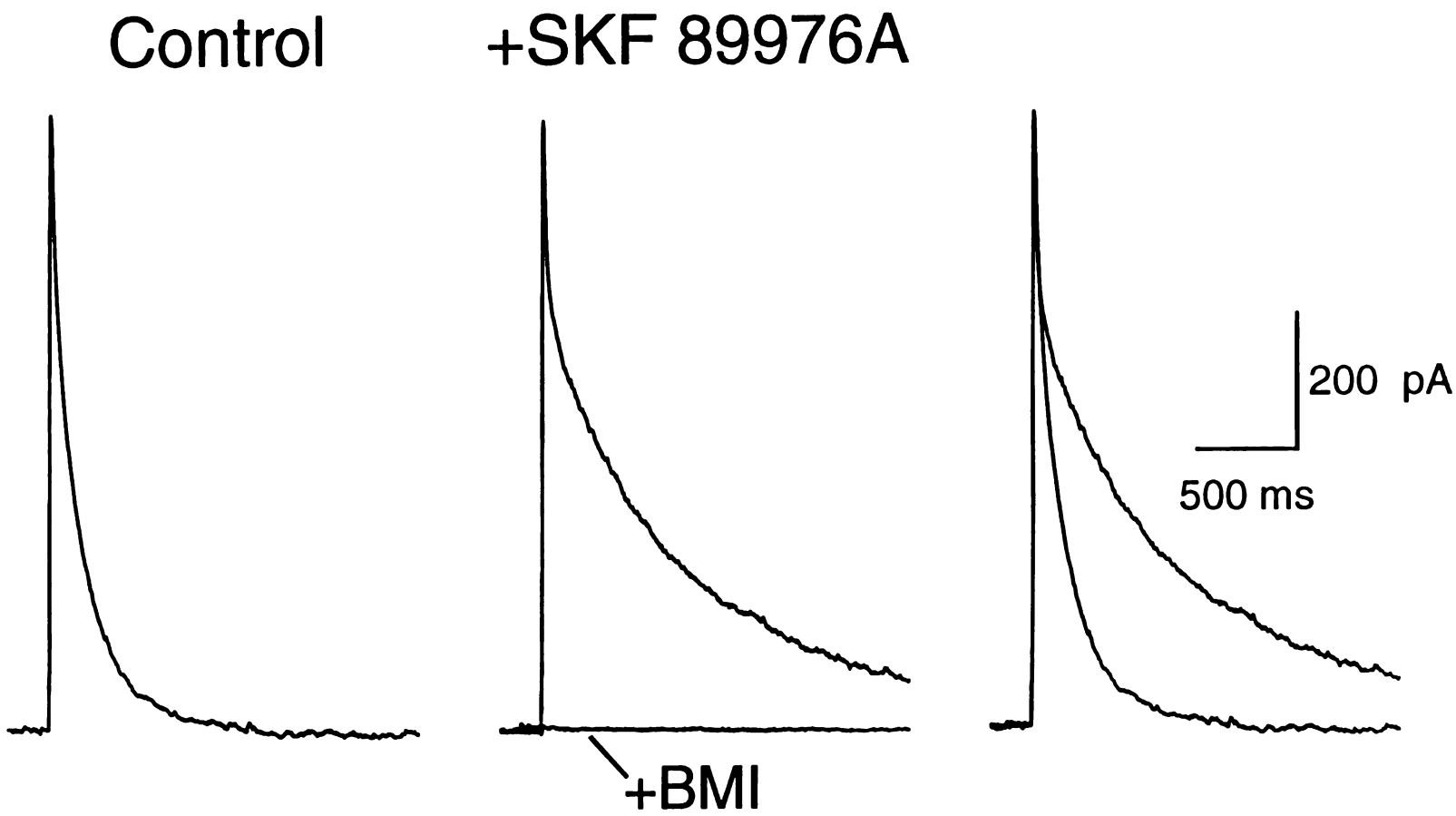

Control
+SKF 89976A
200 pA
500 ms
+BMI

Fig. 8 SKF 89976A prolongs the time course of large monosynaptic $GABA_A$ IPSCs evoked with strong stimulation but has little effect on small synaptic currents evoked with weak stimulation. Strong and weak monosynaptic IPSCs were recorded from a CA1 neuron (Vh = -50 mV) and evoked by alternating the stimulus strength of a bipolar electrode placed in stratum pyramidal from 14 V to 4 V. *Top,* weak stimulation evokes a small IPSC which appears relatively unchanged after addition of SKF 89976A (20 uM) to the slice. The responses before and after treatment with the drug are shown superimposed to the right. *Bottom,* in the same cell a strong stimulus evokes a much larger IPSC which is markedly augmented by SKF 89976A. The IPSC recorded in the presence of the drug is superimposed over the control response.

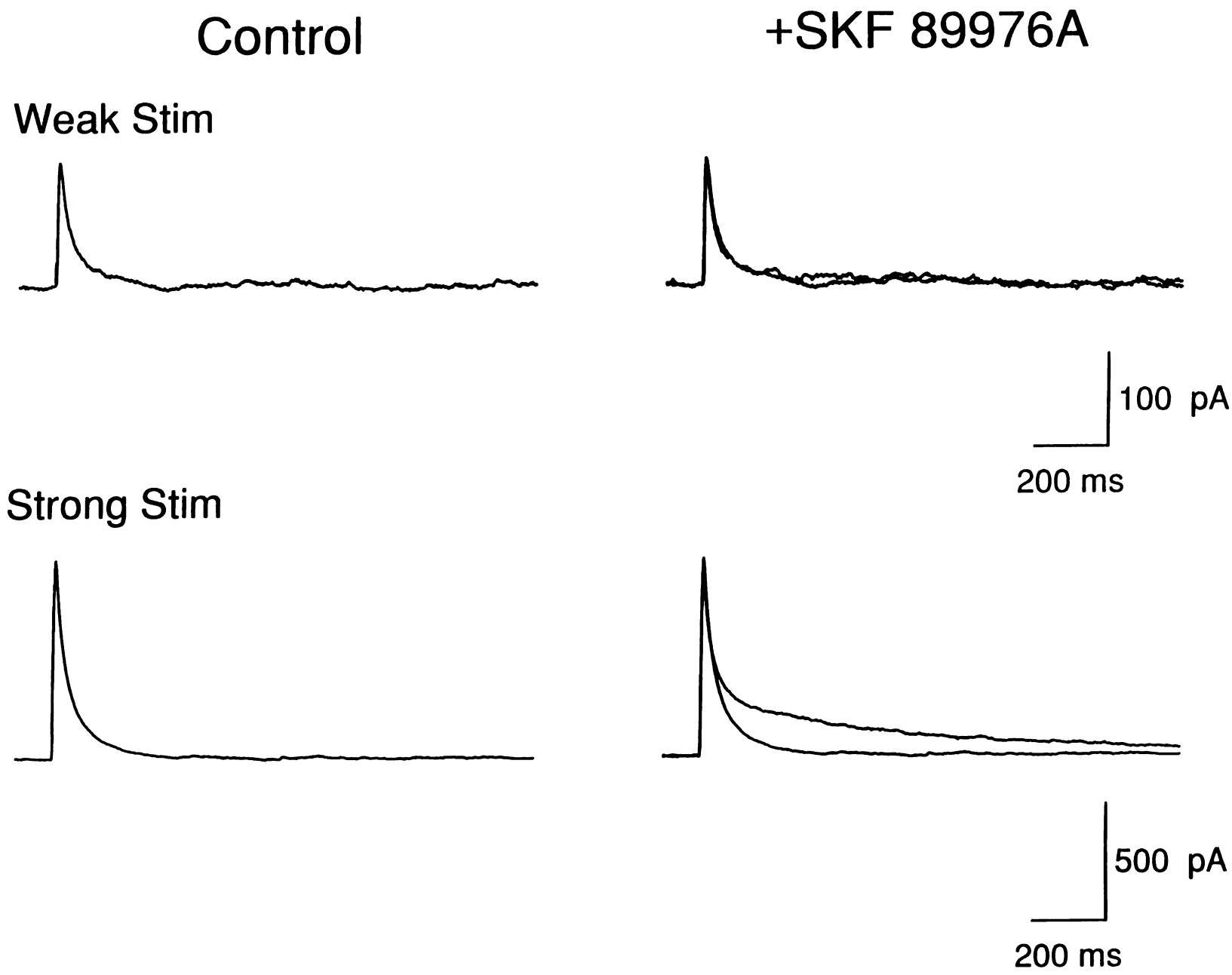

Control
+SKF 89976A
Weak Stim
100 pA
200 ms
Strong Stim
500 pA
200 ms

Fig. 9 Spontaneous IPSCs are not affected by SKF 89976A. Monosynaptic $GABA_A$ IPSCs were recorded from a voltage-clamped CA1 cell (CsCl-based internal solution, Vh = -70 mV). *Top*, 100 consecutive spontaneous IPSCs were aligned and averaged before and after the addition of SKF 89976A (25 μM) to the bath. The time course of the spontaneous events were not obviously affected by the drug and the averaged records obtained before and after SKF 89976A are superimposed to the right. *Insets*, representative spontaneous IPSCs used to construct the averaged records (cal. = 20 pA/50 ms). *Bottom*, stimulus-evoked IPSCs from the same cell are prolonged by SKF 89976A. Monosynaptic IPSCs were evoked from a bipolar stimulating electrode placed in the pyramidal cell layer. SKF 89976A had its typical effect of enhancing the time course of the evoked synaptic current and the responses collected during the same periods as above are superimposed to the right.

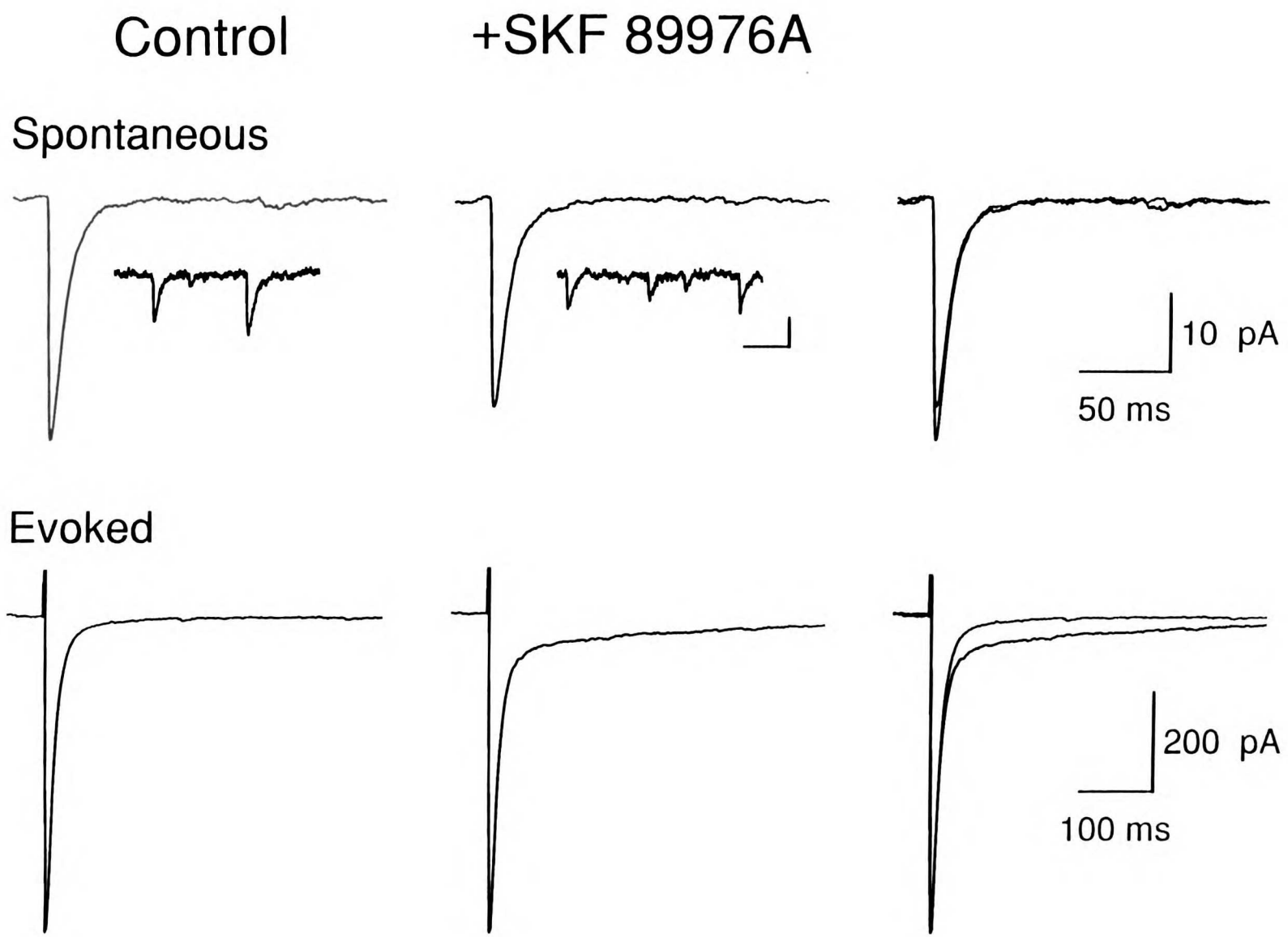

Control
+SKF 89976A
Spontaneous
10 pA
50 ms
Evoked
200 pA
100 ms

Fig. 10 Stimulus-evoked transmitter levels in the slice are enhanced by SKF 89976A. A CA1 outside-out patch (Vh=-70 mV) was positioned at the surface of the pyramidal cell layer, close to a bipolar stimulating electrode. *Left*, paired pulse stimulation (20 ms interval) reproducibly elicited $GABA_A$-receptor channel activity in the patch. In this and the remaining panels, three representative responses are shown above the ensemble average of 10-15 consecutive episodes. *Middle*, shortly after its addition to the superfusing solution, SKF 89976A (20 uM) greatly increased the stimulus-evoked response. *Right*, stimulus-evoked channel activity in the patch was sharply reduced by moving the electrode 150 μm away from the slice. Channel activity could be restored by moving the electrode back to its original position (not shown).

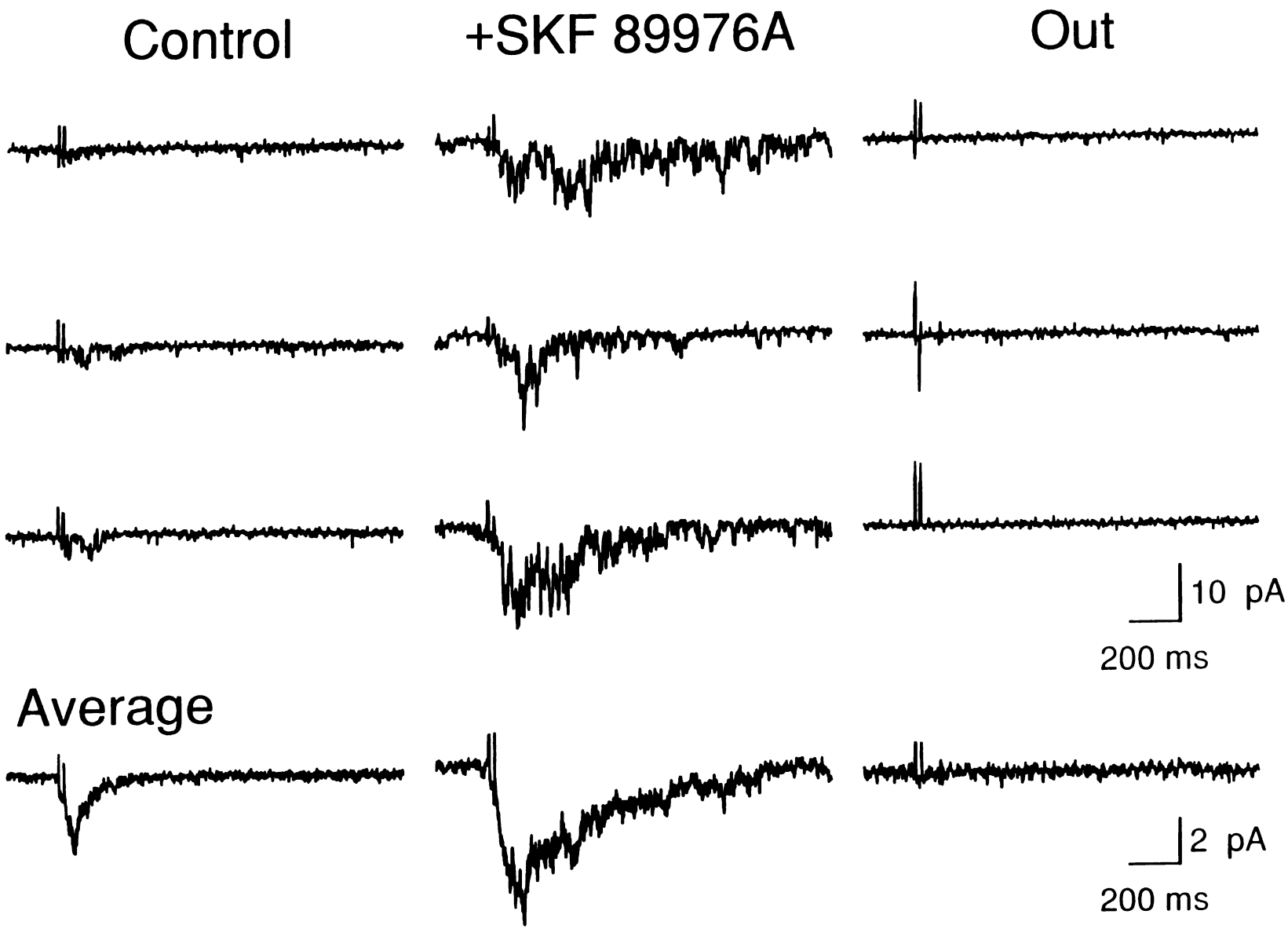
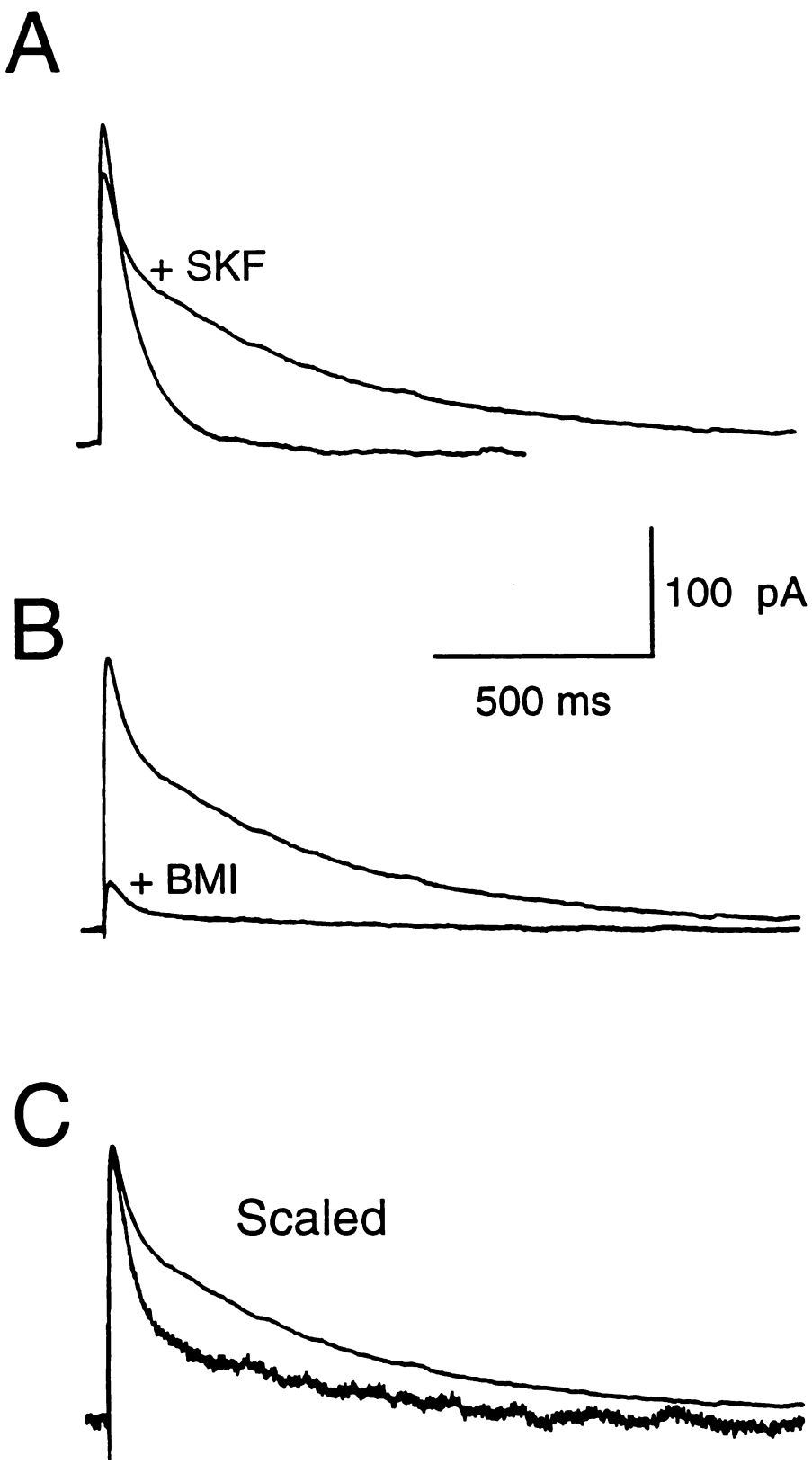

Control
+SKF 89976A
Out
10 pA
200 ms
Average
2 pA
200 ms

Fig. 11 The competitive antagonist BMI speeds the rate of decay of the $GABA_A$-mediated IPSC in the presence of SKF 89976A. Monosynaptic fast IPSCs were recorded in the presence of CGP 35348 (500 μM) in a voltage-clamped pyramidal cell (Vh = -55 mV). *A*, SKF 89976A (20 μM) was added to the superfusing solution and had its typical effect to prolong the $GABA_A$ IPSC. B, a low concentration of BMI (5 μM) was then added to the bathing solution and caused a marked reduction in the amplitude of the synaptic current. The reduction in the amplitude of the IPSC was associated with an increase in the speed of recovery of the synaptic current. *C*, this effect on the time course of the IPSC is more clear when the traces shown in B are scaled to the same amplitude.


A
+ SKF
100 pA
B
500 ms
+ BMI
C
Scaled

The influence of GABA-uptake on the time course of fast and slow inhibitory synaptic transmission

II. Presynaptic inhibition

Introduction

In addition to their postsynaptic actions, many neurotransmitters and their agonists have powerful presynaptic effects (Nicoll et al., 1990; Starke, 1981). In the CNS, receptors on nerve terminals are widespread, but in most cases the physiological role of these receptors is unclear since it is not known if synaptically-released transmitters gain access to these sites. The best characterized role for presynaptic receptors comes from studies on spinal presynaptic inhibition, in which axo-axonic synapses release GABA onto primary afferent fibers. This synaptically-released GABA acts primarily through an action on $GABA_A$ receptors to inhibit transmitter release from the afferent terminals (Eccles, Schmidt & Willis, 1963; Stuart & Redman, 1992). It is generally agreed, however, that axo-axonic synapses on synaptic terminals are confined to the brain stem and spinal primary afferent fibers (Peters, Palay & Webster, 1991). Another way, in addition to axo-axonic synapses, in which neurotransmitters have direct access to presynaptic receptors is at terminals from which they themselves have been released (autoreceptors) (Langer, 1987; Starke, 1981; Starke, Gothert & Kilbinger, 1989). A third possibility is that released neurotransmitters may act on distant synapses in a paracrine fashion (Fuxe & Agnati, 1991). Indeed, many instances have been found in which the neurotransmitter receptor distribution does not match the distribution of neurotransmitter (Herkenham, 1987). However, there is little physiological evidence in the CNS in support of the distant action of synaptically-released neurotransmitters.

As discussed in the preceding chapter, in the hippocampus, synaptically-released GABA generates both fast ($GABA_A$-receptor mediated) and slow ($GABA_B$ receptor-mediated) postsynaptic inhibition. In addiition to this postsynaptic inhibition, application of the $GABA_B$ agonist, baclofen, can block both excitatory and inhibitory synaptic transmission by activating presynaptic receptors (Ault & Nadler, 1982; Bowery et al., 1980; Dutar & Nicoll, 1988b; Harrison, 1990; Inoue et al., 1985; Lanthorn & Cotman, 1981; Olpe et al., 1982). While GABA regulates its own release locally via autoreceptors on inhibitory terminals (Davies et al., 1990; Diesz & Prince, 1989; McCarren & Alger, 1985), it is unclear whether GABA can diffuse to the presynaptic receptors on excitatory nerve terminals. We have designed experiments to address this issue as well as to explore the role of uptake in controlling the presynaptic actions of GABA.

Results

We initially attempted to determine if synaptically-released GABA activates presynaptic $GABA_B$ receptors on excitatory terminals in the hippocampus by examining the actions of the selective $GABA_B$ receptor antagonist CGP 35348 on excitatory synaptic responses. However, the antagonist had no consistent effects on single synaptic responses evoked by stimulation in the dendritic region of CA1 pyramidal cells, nor did it affect responses evoked with a paired pulse protocol similar to that used to study the autoinhibition of inhibitory synaptic responses (Davies et al., 1990). Nevertheless, a single conditioning stimulus may not have released enough GABA to produce the effect or paired pulse stimulation may have produced a presynaptic enhancement of synaptic strength that obscured the inhibition.

We then designed an experiment to study the action of GABA, released from a repetitively-stimulated set of fibers, on a naive set of excitatory afferent inputs. As illustrated in Fig. 1A, two stimulating electrodes which activated overlapping but independent excitatory fibers were placed on either side of the recorded cell. One electrode (S_1) was used to deliver a brief tetanus (5 pulses, 50 Hz) that would activate both excitatory and inhibitory fibers in the hippocampal slice. The other electrode (S_2) was used to evoke single excitatory postsynaptic currents (EPSCs). Whole-cell recording was used to record the synaptic events and the pipette solution contained Cs^+ to minimize the postsynaptic contribution of the slow inhibitory postsynaptic current (IPSC). When the two stimulating electrodes were properly positioned, a brief tetanus to S_1 reliably reduced the size of the EPSC evoked by electrode S_2 (Fig. 1B). To determine if $GABA_B$ receptors were involved in this inhibition, the selective $GABA_B$ antagonist CGP 35348 (Olpe et al., 1990) was added to the solution superfusing the slice. Shortly

after its addition, the antagonist abolished the inhibition of the test response, indicating that $GABA_B$ receptors mediated the depression of excitatory synaptic transmission.

The time course of the inhibition, examined by varying the interval between S_1 and S_2, is shown by the closed circles in Fig. 2. The inhibition peaks at about 300 ms and lasts for more than one second. The time course of a typical $GABA_B$-mediated slow IPSC, evoked with a brief tetanus in a cell in the absence of Cs^+ in the recording pipette, is shown on the same graph. The time course of the postsynaptic $GABA_B$ response was quite constant as shown by the average time course of the slow IPSC derived from recordings in four cells (diamonds). Although the time courses of the pre-and postsynaptic actions of GABA are similar, the inhibition of EPSCs reaches its maximum somewhat later than does the slow IPSC and lasts for a longer time. The inhibition of the EPSCs was entirely blocked by CGP 35348 (open circles).

The inhibition of excitatory transmission was also observed using field potential recording of excitatory postsynaptic potentials (EPSPs). In these experiments, the same experimental design was used as in Fig. 1A but the extracellular recording electrode was placed midway between the two stimulating electrodes in s. radiatum. The stimulation protocol is shown schematically in Fig 3A. To establish firmly that the depression was indeed presynaptic, paired pulse facilitation was monitored by delivering pairs of stimuli separated by 50 ms. If the inhibition is presynaptic the degree of facilitation should increase, as with other manipulations that decrease transmitter release (Harris & Cotman, 1983; Katz & Miledi, 1968; Mallart & Martin, 1968; Muller & Lynch, 1989). Fig. $3B_1$ shows that when preceded by a conditioning tetanus, the test EPSP is reduced (filled circles) compared to the test EPSP alone (open circles) and that this difference is abolished by

application of CGP 35348. As shown in Fig. $3B_2$ paired-pulse facilitation, expressed as a facilitation ratio, was also monitored in this experiment and increased during the inhibition. As with the inhibition, the change in the facilitation ratio was blocked by CGP 35348. Sample records from this experiment are shown in Fig 3C.

Although the $GABA_B$-mediated presynaptic inhibition is clearly present at 38° C (Fig. 1), the inhibition was more robust at lower temperatures (30-32° C), facilitating characterization of the effect, and suggesting that transmitter uptake mechanisms, which are strongly temperature-sensitive (e.g.(Iversen & Neal, 1968), might be involved. If the inhibition depends upon the spread of GABA over some distance, one might expect that GABA-uptake would play an important role in controlling this presynaptic action. We therefore examined the effect of the blockade of GABA-uptake on this inhibition with SKF 89976A, a competitive inhibitor of the GABA transporter (Falch et al., 1990; Larsson et al., 1988).

As discussed previously, SKF 89976A markedly enhanced the postsynaptic $GABA_B$ response generated from synaptically-released transmitter (preceding chapter). These monosynaptically-evoked slow IPSCs were generated in response to single pulses of stimuli, however, and it is unclear if uptake influences postsynaptic $GABA_B$ responses generated by the larger amount of transmitter release induced by repetitive stimulation. We next studied the effect of SKF 89976A on postsynaptic $GABA_B$ responses evoked with a repetitive stimulation paradigm that was similar to the protocol used to evoke heterosynaptic depression. In accord with its actions on IPSCs evoked with single stimuli, SKF 89976A both increased the amplitude and prolonged the time course of slow IPSCs evoked with

repetitive stimulation. Fig. $4A_1$ shows a representative example of the effect of the uptake-blocker on a slow IPSC evoked by a brief tetanus.

To examine whether the inhibition of uptake effects the presynaptic inhibititory effect of GABA, we made use of the similar time course of the slow IPSC and of the inhibition of excitatory transmission (Fig 2). Thus, in the next series of field EPSP experiments, the conditioning stimulus consisted of fewer pulses than used previously (3 stimuli, 50 Hz) and the interval between the conditioning tetanus and test stimulus was set at 700-800 ms (Fig. $4A_2$). This long interval was chosen to reduce the degree of presynaptic inhibition in control conditions (Fig. 2). Since SKF 89976A greatly prolonged the slow IPSC (Fig. $4A_1$), it might be expected that the presynaptic inhibitory effect of GABA at this interval would be enhanced as well. As expected at this interval and with a briefer tetanus, a preceding conditioning tetanus (S1) had only a small effect on the test response (S2) (filled circles) as shown during the control period in Fig. $4B_1$, which is the average of 6 separate experiments. In the presence of SKF 89976A, however, a marked depression of the test response occurred (Fig. $4B_1$ and C_2) which was associated with a clear increase in paired pulse facilitation (Fig.$4B_2$). The subsequent addition of CGP 35348 to the superfusing solution abolished the depression (Fig. $4B_1$ and C_3) and the change in paired pulse facilitation (Fig. $4B_2$). These results suggest that some "spill-over" of GABA onto the terminals of excitatory synapses occurs normally, an effect that is greatly enhanced after blockade of GABA-uptake.

Previous experiments examining the effect of SKF 89976A on fast somatic postsynaptic inhibition indicated that transmitter spill-over only became apparent when uptake was inhibited (previous chapter). However, spill-over of GABA onto presynaptic receptors occurs under normal

conditions in the dendritic region of the slice. One possibility is that GABA-uptake is more effective at the soma vs. dendrites of pyramidal cells. Indeed, when the actions of SKF 89976A on the responses to GABA applied to the dendritic region and to the cell body region of the slice were compared, we found that the somatic responses were potentiated to a far greater extent than those evoked in the dendrites (n = 4) (Fig. 5). This result suggests that high-affinity GABA-uptake is less powerful in the dendritic region of the slice.

Discussion

Presynaptic receptors for a number of transmitters including GABA, acetylcholine, adenosine, norepinephrine, and numerous peptides mediate the inhibition of excitatory synaptic transmission (Kandel et al., 1991; Nicoll et al., 1990). Although the presynaptic actions of these transmitters have been typically characterized by exogenously-applied agonists, little is known about their physiological importance. Specifically, whether or not synaptically-released transmitters gain access to the receptors at these sites has not been clearly established.

One hindrance to establishing a physiological role for presynaptic receptors is the general lack of anatomical evidence for axo-axonic synapses in the brain. How then, without a directly-situated delivery site, do these neurotransmitters reach their receptors? Anatomical studies of cortical neurons have shown that GABA-releasing terminals form conventional synapses primarily onto the dendritic shafts and somata of target cells (Beaulieu & Somogyi, 1990; Kisvárday, Gulyas, Beroukas, North, Chubb & Somogyi, 1990), and less frequently, make synapses onto dendritic spines (Beaulieu & Somogyi, 1990; Fifková, Eason & Schaner, 1992), the site at which excitatory afferent fibers terminate. It seems reasonable then that transmitter released from these conventional synapses could "spill-over" and act as a diffuse signal onto nearby (within microns) excitatory synaptic terminals.

In this study, we have provided evidence for this hypothesis by showing that repetitive stimulation of one bundle of afferent fibers in the s. radiatum region of CA1 causes a marked short-lasting, heterosynaptic depression of excitatory transmission in an independent, overlapping set of inputs. This inhibition is due to the activation of presynaptic $GABA_B$ receptors, since it is accompanied by an increase in paired pulse facilitation

and is blocked by the selective $GABA_B$ antagonist CGP 35348. In addition to the autoinhibition of GABAergic transmission (Davies et al., 1990), this result indicates that GABA, released by brief, repetitive stimulation of inhibitory afferent fibers, can act trans-synaptically to inhibit excitatory synaptic transmission (Fig. 6). These results clearly provide a physiological role for neurotransmitter inhibition via presynaptic receptors on excitatory terminals in the hippocampus.

The time course of the $GABA_B$-mediated presynaptic inhibition was somewhat different from that of the $GABA_B$-mediated postsynaptic current. The presynaptic effect peaked later and lasted longer than the slow IPSC. A similar difference was found between the time course of the slow IPSP and the autoinhibition of IPSCs in the hippocampus (Davies et al, 1990). As suggested previously (Davies et al., 1990), the difference between pre- and postsynaptic $GABA_B$ responses may arise in a number of ways. Presynaptic receptors may differ from their postsynaptic counterparts in their affinity for transmitter or perhaps in their coupling to different G protein-mediated messenger systems. Alternatively, there may be more "spare receptors" on presynaptic terminals than on postsynaptic cells.

To act effectively at a distance, neurotransmitters require high affinity receptors. The affinity of receptors in brain slices cannot be determined since potent uptake mechanisms interfere with studying the sensitivity of receptors to low concentrations of GABA. However, in cultured hippocampal cells where agonist concentrations are better controlled, presynaptic $GABA_B$ receptors were found to be highly sensitive to GABA (Yoon & Rothman, 1991). In these cells, a low concentration of GABA (1 uM) almost maximally attenuated excitatory synaptic currents but had little direct effect on postsynaptic $GABA_A$ receptors. Therefore, a relatively low concentration of

GABA in the dendritic region of CA1 would be sufficient to mediate the presynaptic inhibition of excitatory afferents without having a direct postsynaptic action on the receptors governing fast synaptic inhibition.

By using a dialysis probe *in vivo*, the concentration of GABA in the extracellular fluid of the hippocampus has been estimated to be 0.2 - 0.8 μM (Lerma et al., 1986; Tossman, Jonsson & Ungerstedt, 1986), raising the possiblity that ambient levels of GABA might tonically inhibit excitatory transmission. Our results do not support this possibility since, as has been reported previously (Davies, Starkey, Pozza & Collingridge, 1991), CGP 35348 had no consistent effect on normal excitatory transmission evoked at a low frequency. Ambient levels of GABA are therefore insufficient to tonically activate presynaptic $GABA_B$ receptors. Thus, either the concentrations measured with dialysis techniques do not reflect the concentrations at synaptic locations, or the sensitivity of the excitatory synapses in the slice to GABA is lower than in culture.

Transmitter uptake into neurons and glial cells is a crucial factor in maintaining low ambient levels and limiting the action of a number of transmitters in the brain (Kandel et al., 1991). Transport processes with a high affinity for GABA (K_m's in the nanomolar to low micromolar range for some systems) have been well-characterized in the CNS (Hertz, 1979; Iversen & Kelly, 1975) and a family of genes encoding these transporters has been isolated (Clark, Deutch, Gallipoli & Amara, 1992; Guastella, Nelson, Nelson, Czyzyk, Keynan, Miedel, Davidson, Lester & Kanner, 1990). We have found that SKF 89976A, a competitive inhibitor which is equally effective upon neuronal and glial GABA-uptake (Falch et al., 1990; Larsson et al., 1988) dramatically enhanced the magnitude of the GABA-mediated inhibitition of

excitatory transmission, indicating that transmitter uptake actively regulates the diffuse inhibitory synaptic action of GABA.

In contrast to its action on GABA released transiently by synaptic stimulation, SKF 89976A, applied for ten to fifteen minutes, had no obvious effect on normal excitatory transmission evoked at a low frequency (0.1-0.033 Hz) or on the resting membrane potential, suggesting that blocking uptake does not raise ambient GABA to levels sufficient to activate either pre- or postsynaptic GABA receptors. The uptake blocker nipecotic acid, which elevates extracellular GABA via heteroexchange (Johnston et al., 1976; Solís & Nicoll, 1992b; Szerb, 1982) has been recently shown to inhibit evoked excitatory synaptic potentials in the neostriatum by a bicuculline-resistant action (Calabresi, Mercuri, De Murtas & Bernardi, 1991), most likely by increasing the ambient level of GABA.

Why does "spill-over" occur onto presynaptic $GABA_B$ receptors but not onto neighboring $GABA_A$ synapses when uptake is intact? Two possibilities come to mind. First, as discussed above, the $GABA_B$ receptors may have a higher affinity than the $GABA_A$ receptors. Second, the extremely high density of inhibitory terminals on the soma and initial segment of CA1 pyramidal neurons (Somogyi et al., 1983) results in a high density of GABA transporters as demonstrated by immunocytochemistry (Radian, Ottersen, Storm-Mathisen, Castel & Kanner, 1990) and a more effective removal of GABA compared to dendritic sites (Fig. 5) (Rovira, Ben-Ari & Cherubini, 1984) where inhibitory synapses are less concentrated. These findings raise the possibility that the area over which a transmitter can act, and perhaps also the local concentration of transmitter, may vary in different regions depending on the local uptake processes.

Taken together, these results suggest that GABA released from inhibitory terminals can diffuse to neighboring presynaptic $GABA_B$ receptors and exert a profound depression of excitatory transmitter release. GABA-uptake, by modulating the effective spread of this diffuse inhibition, may therefore directly influence the strength of excitatory synaptic transmission in the hippocampus. As described in the previous chapter, uptake governs the strength of slow postsynaptic inhibition and has a strong influence on fast transmission. It serves to localize fast $GABA_A$-mediated transmission by preventing its spill-over to neighboring synapses. Thus, conditions which compromise uptake mechanisms augment diffuse transmitter actions at the expense of point-to-point synaptic transmission.

Figures

Fig. 1 $GABA_B$ receptors mediate a short-lasting heterosynaptic depression of excitatory synaptic transmission. (*A*) Schematic diagram illustrating the placement of stimulating and recording electrodes used to study heterosynaptic depression. One stimulating electrode (S1), that activates excitatory afferent fibers as well as inhibitory fibers from local interneurons (I), was used to deliver a brief conditioning tetanus (5 pulses, 50 Hz). This conditioning stimulus was delivered 200 ms before the stimulation of a seperate set of test inputs from electrode S2. (*B*) Heterosynaptic depression in a voltage-clamped cell (V_h= -80 mV, temperature = 38° C) is blocked by CGP 35348. (*Top)* test EPSCs alone (S2) and preceded by a conditioning tetanus (S1). Both traces are shown superimposed on the right. The conditoning tetanus caused a clear reduction in the amplitude of the test EPSC. (*Bottom*) this effect was blocked by the subsequent addition of CGP 35348 (500 μM) to the superfusing medium.

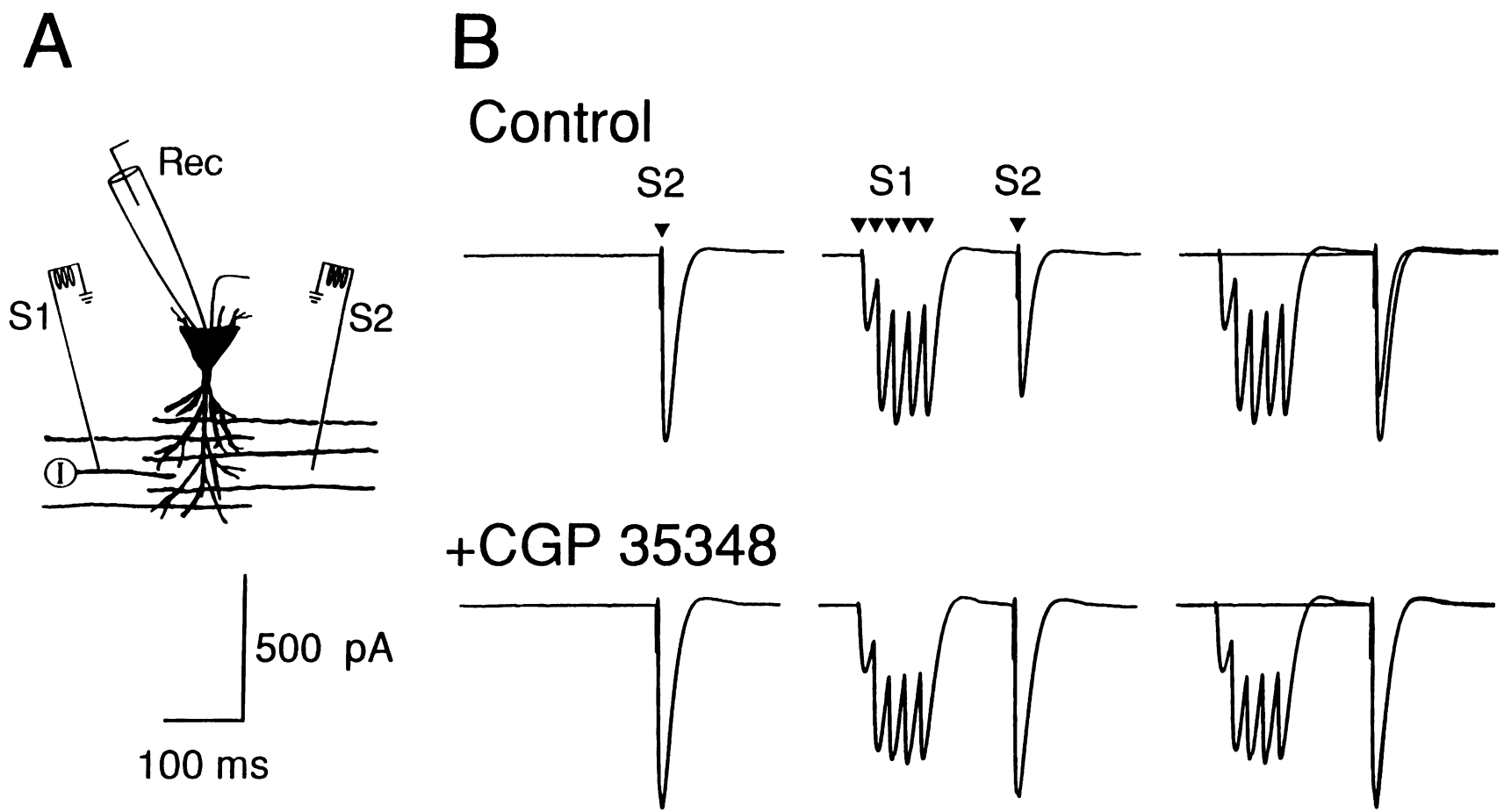

A
Rec
S1
S2
500 pA
100 ms
B
Control
S2
S1
S2
+CGP 35348

Fig. 2 The time course of the heterosynaptic depression is similar to the time course of the slow IPSC. The time course of heterosynaptic depression was studied by varying the interval between the conditioning tetanus (5 pulses, 50 Hz) and the test stimulus. The amount of depression is plotted as the amplitude of the test pulse when preceded by the conditioning tetanus relative to the amplitude of the test pulse alone (filled circles, n = 9 cells). In four of the cells, CGP 35348 was added to the superfusing solution. The $GABA_B$ antagonist completely blocked the depression at the six intervals tested (open circles). A monosynaptic slow $GABA_B$ IPSC was evoked with a brief tetanus (5 pulses, 50 Hz) in a cell recorded with K^+ in place of Cs^+ in the patch electrode and with 20 μM CNQX added to the superfusing solution. The trace is shown inverted for comparison to the time course of the heterosynaptic depression. The peak amplitude of the slow IPSC was 60 pA. Superimposed on the individual trace is the average time course of the slow IPSC derived from four cells (diamonds) plotted relative to their peak amplitude. The slow IPSCs were normalized with respect to their peak amplitudes before averaging.

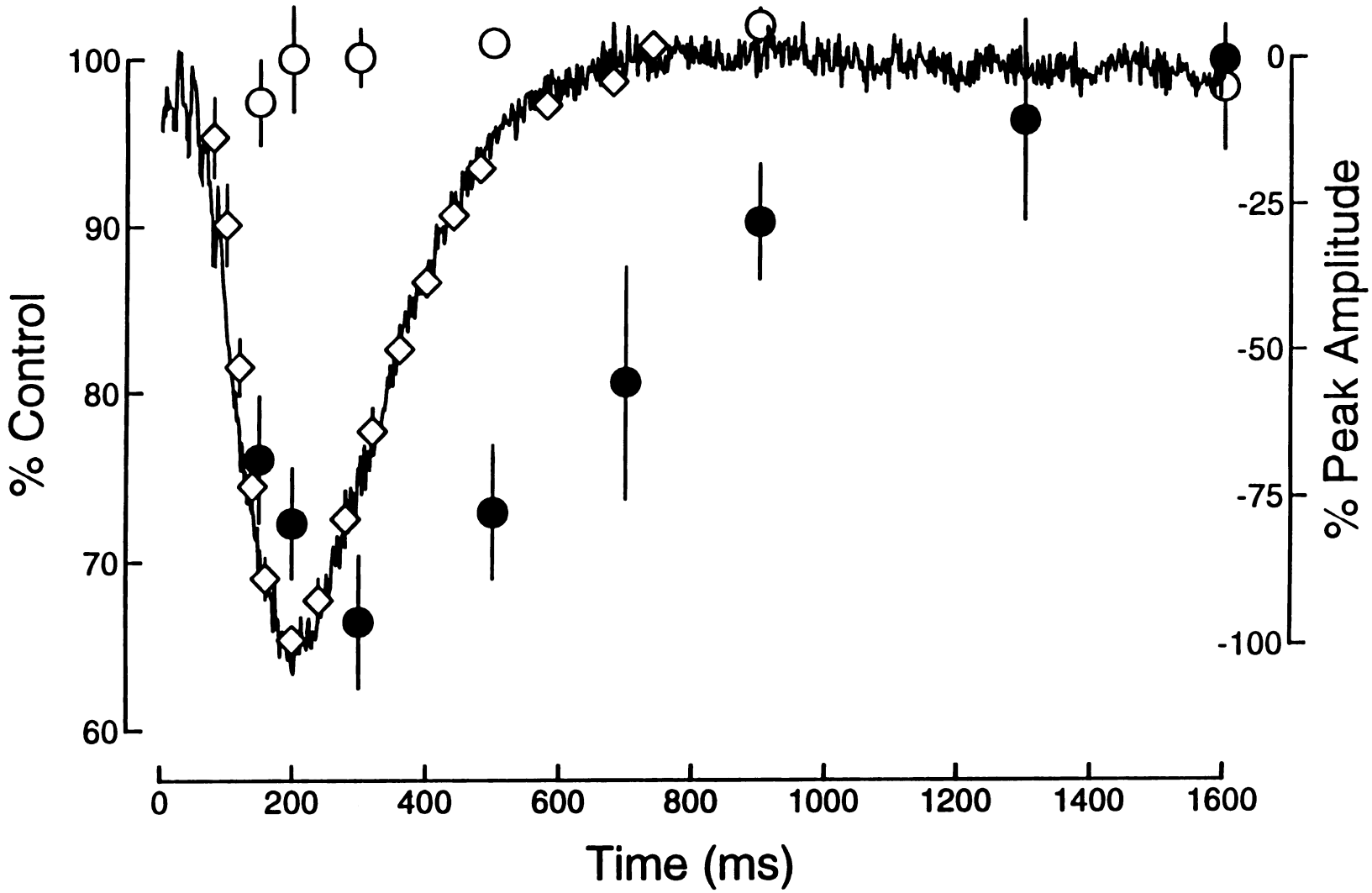

100
90
80
70
60
% Control
0
-25
-50
-75
-100
% Peak Amplitude
0
200
400
600
800
1000
1200
1400
1600
Time (ms)

Fig. 3 $GABA_B$ receptor-mediated heterosynaptic depression of the field EPSP is accompanied by an increase in paired pulse facilitation. (*A*) Stimulation protocol used to study heterosynaptic depression. Test EPSPs were evoked in one pathway (S2) with paired pulse stimulation (ISI = 50 ms) every 15 sec. Test responses were evoked with (filled circles) or without (open circles) a preceding conditioning tetanus delivered to an overlapping pathway (S1). (B_1) Stimulation of the conditioning pathway caused a decrease in slope of the test response. This inhibition was quickly reversed following the addition of CGP 35348 (500 μM) to the superfusing medium for the period marked by the bar. (B_2) The depression of the test response was accompanied by an increase in the amount of paired pulse facililitation, calculated as the ratio of the slope of the second pulse to that of the first. This increase in facilitation ratio was completely abolished by the addition of CGP 35348 to the superfusing solution. Each point represents the average value for three consecutive responses. (C) Representative field EPSPs preceded by the conditioning tetanus are superimposed upon the alternately evoked test EPSPs before (C_1) and after (C_2) the addition of CGP 35348. Traces are averages of 5-10 responses from the periods marked in B_1.

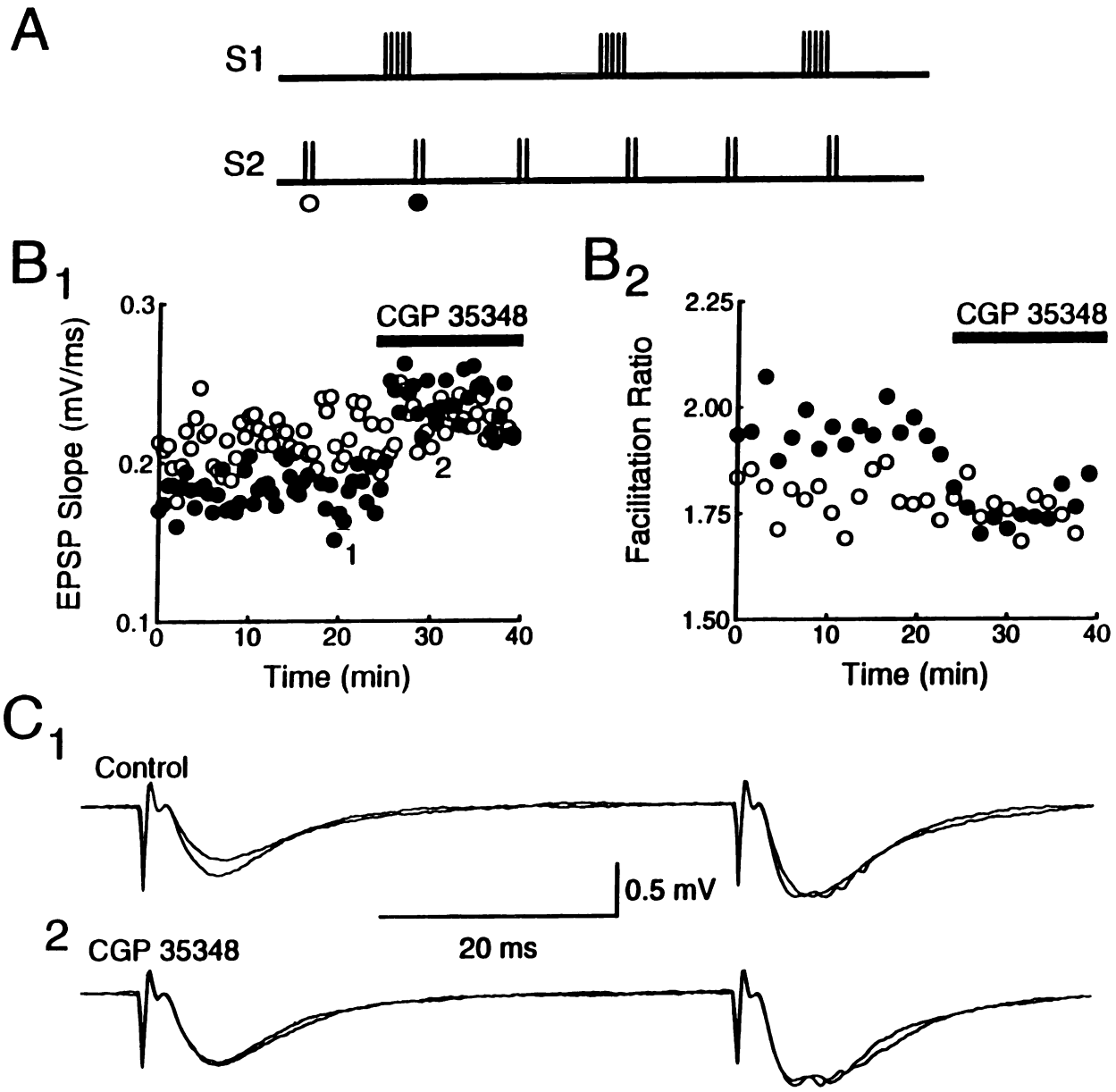

A
S1
S2
B1
EPSP Slope (mV/ms)
0.3
0.2
0.1
CGP 35348
1
2
0
10
20
30
40
Time (min)
B2
Facilitation Ratio
2.25
2.00
1.75
1.50
CGP 35348
0
10
20
30
40
Time (min)
C1
Control
0.5 mV
20 ms
2
CGP 35348

Fig 4. SKF 89976A enhances both the pre-and postsynaptic actions mediated by $GABA_B$ receptors in the slice. (A_1) A monosynaptic $GABA_B$-mediated IPSC from a voltage-clamped CA1 cell (V_h=-60 mV) evoked with a brief tetanus (5 pulses, 50 Hz) before and after the addition of SKF 89976A (25 μM) to the superfusing solution. The uptake inhibitor increased the peak amplitude of the slow IPSC and greatly prolonged its decay. (A_2) A representative trace of field EPSPs illustrating the protocol used to study the influence of GABA-uptake on presynaptic inhibition. In this series of experiments, the conditioning tetanus (S1, 3 pulses at 50 Hz) is delivered 700 - 800 ms before the test stimuli (S2). (*B*) SKF 89976A enhances the inhibition of the field EPSP and increases paired pulse facilitation. Control test stimuli (*open circles*) were evoked every 30 seconds, alternately accompanied by a preceding conditioning tetanus (*filled circles*). The field EPSP slope values were normalized to the average of the first 20 contol test stimuli. The plotted points represent the mean +/- s.e.m. from six slices. (B_1) Initially, at these long intervals the conditioning tetanus had little effect on the slope of the test stimulus. SKF 89976A (25 μM), added to the bath during the period marked by the bar, caused a 25 % decrease in the slope of the test pulse evoked with a preceding conditioning tetanus. This inhibition of the field EPSP was rapidly blocked following the subsequent addition of CGP 35348 (500 μM) to the superfusing medium during the time indicated by the dashed line. The slope of the control test stimuli was not affected. (B_2) The facilitation ratios of the test stimuli preceded by the conditioning tetanus were normalized and averaged as in B_1. SKF 89976A caused a marked increase in the facilitation ratio which was completely reversed by CGP 35348. The facilitation ratio of the test EPSP evoked without the conditioning tetanus was not affected by the uptake inhibitor or receptor antagonist (not shown) .(C_{1-3}) Representative

traces of the test stimuli preceded by a conditioning tetanus from one experiment corresponding to the periods indicated in B_1.

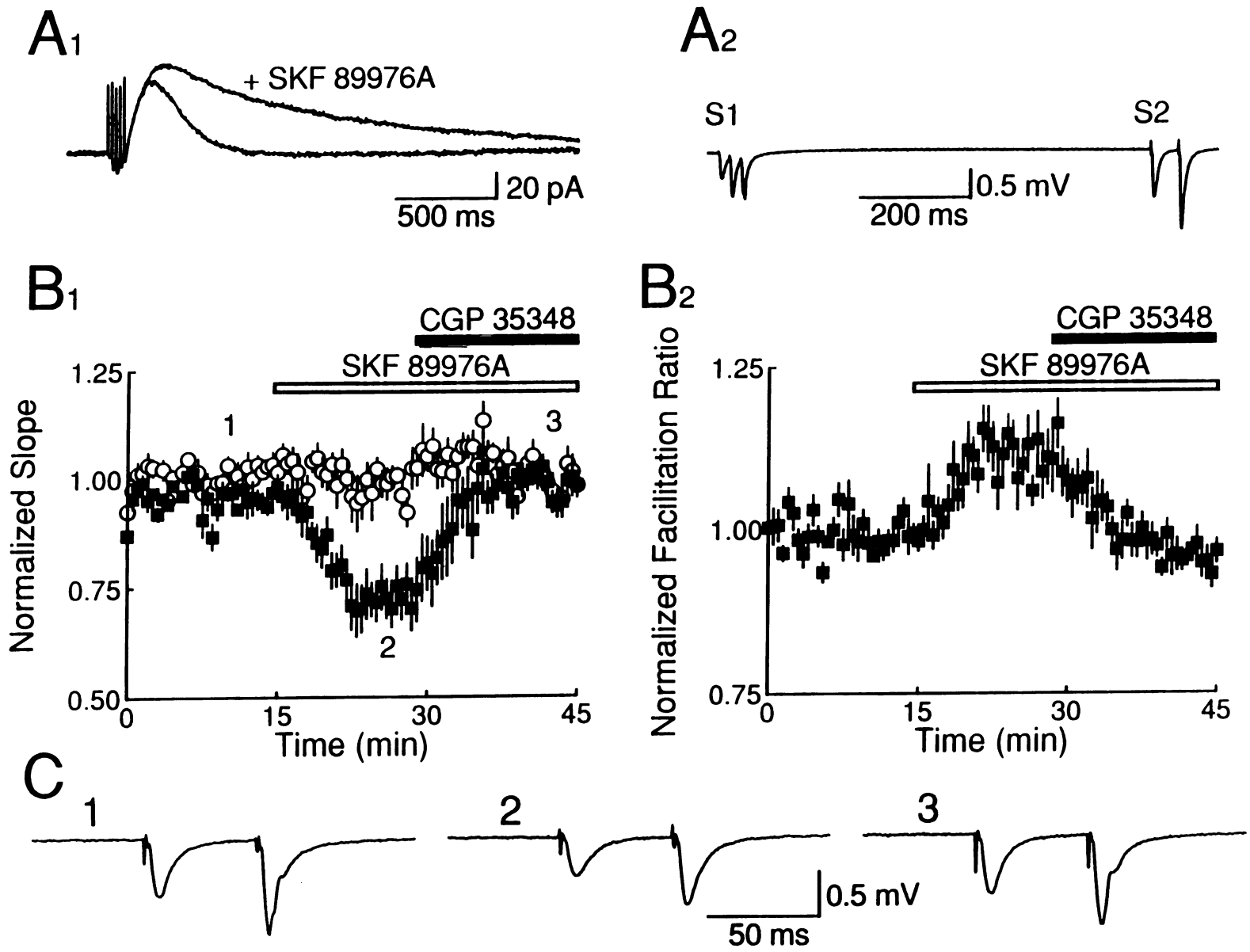
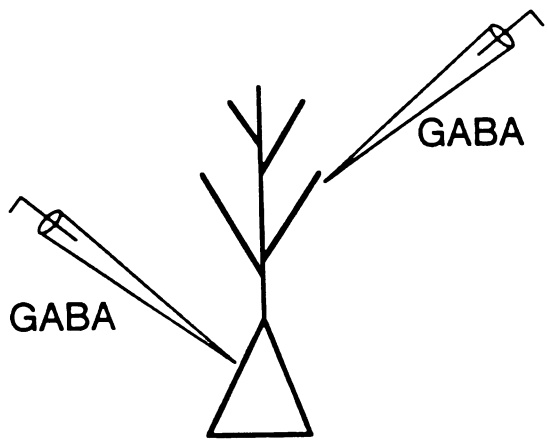

A1
+ SKF 89976A
20 pA
500 ms
A2
S1
S2
0.5 mV
200 ms
B1
CGP 35348
SKF 89976A
Normalized Slope
1.25
1.00
0.75
0.50
1
2
3
0
15
30
45
Time (min)
B2
CGP 35348
SKF 89976A
Normalized Facilitation Ratio
1.25
1.00
0.75
0
15
30
45
Time (min)
C
1
2
3
0.5 mV
50 ms

Fig. 5 The GABA-uptake inhibitor SKF 89976A has a greater potentiating effect on GABA responses evoked in the cell layer vs. dendrites of the slice. Record of membrane current from a voltage-clamped CA1 cell (Vh = -70 mV). GABA was alternately applied to the soma (● , 100 nA/1s) and apical dendrites (○ , 70 nA/1s) via iontophoretic electrodes positioned in stratum pyramidale and stratum radiatum, respectively. SKF 89976A (20 uM) caused a marked increase in the amplitude of the responses to GABA applied at the soma, but had less of an effect on the responses to GABA generated in stratum radiatum.


GABA
GABA

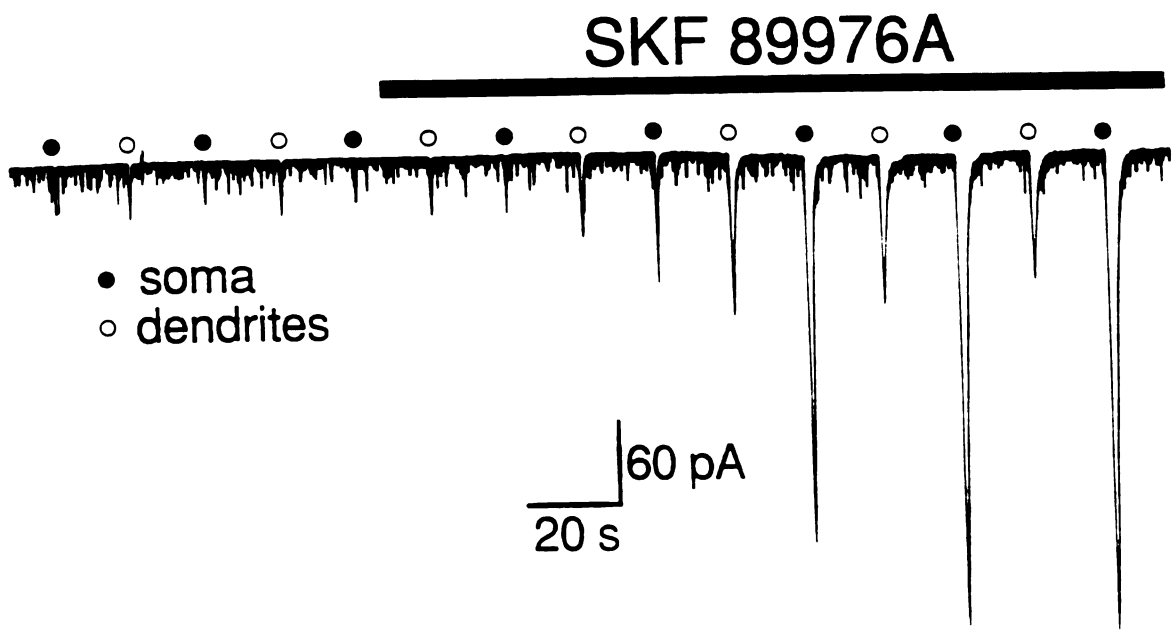
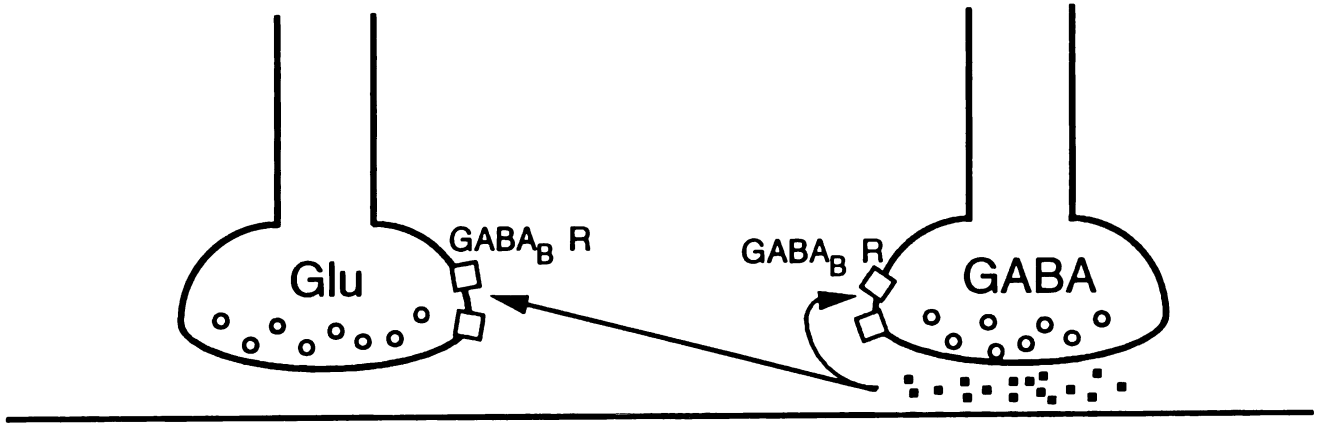

SKF 89976A
• soma
◦ dendrites
60 pA
20 s

Fig. 6 Simplified diagram illustrating the presynaptic actions of GABA in the hippocampus. Synaptically-released GABA can activate $GABA_B$ autoreceptors to inhibit its own release (Davies et al., 1990) and can also act via $GABA_B$ receptors, in a paracrine fashion, to inhibit transmitter release from neighboring excitatory nerve terminals.


Glu
GABA$_B$ R
GABA$_B$ R
GABA

Conclusion

Our understanding of synaptic transmission in the brain has been rapidly advancing on a number of different levels. A great wealth of information has accumulated concerning the physiological properties of the postsynaptic receptors underlying synaptic transmission. Similarly, a considerable effort has been made to understand the presynaptic processes responsible for transmitter release. Much less is known, however, about the behavior of the synaptically-released transmitters themselves, in particular, their effective lifetimes and the spatial domains over which they can act.

Our results and the work of others on fast excitatory transmission in the hippocampus agree with the model originally proposed for the NMJ, that a transient rise in transmitter concentration generates a synaptic current whose decay is governed by the intrinsic kinetics of the postsynaptic receptors. Diffusion of the transmitter from the cleft presumably occurs much faster than the decay of the synaptic current. However, whether the decay of the synaptic current is due completely to the dissociation and closing rate of the receptors, or in part by the rapid entry of glutamate receptors into a desensitized state is uncertain. The magnitude of the contribution of receptor desensitzation to the current time course at hippocampal synapses remains to be determined.

Unlike the NMJ, the major amino acid neurotransmitter molecules in the brain are not inactivated by rapid enzymatic degradation. Rather, specific carrier molecules transport these transmitters into neurons and glial cells. We have found that transmitter uptake is an important regulator of inhibition in

the hippocampus. It apparently acts to prevent the spill-over and accumulation of GABA from neighboring synapses. It is unclear, however, why inhibitors of glutamate uptake have no effect on excitiatory transmission mediated by NMDA receptors in the hippocampal slice. NMDA receptors have a K_d for glutamate in the low micromolar range, and thus might be expected to be highly sensitive to transmitter that spills-over from synapses in the presence of uptake inhibitors. The development of more potent inhibitors of glutamate uptake may yet reveal an important action of these transporters in keeping excitatory transmission highly localized.

We have also described a novel diffuse action of synaptically-released GABA in the hippocampus. This GABA-mediated presynaptic inhibition of excitatory transmission represents one case in the CNS where the mismatch between receptor and transmitter localization can be explained. The "action at a distance" of transmitter thus applies not only to peptidergic transmitters but to the amino acids, and perhaps catecholamines as well. Indeed, the physiological role of presynaptic receptors in many regions of the brain where discrete axo-axonic synapses are not found may be accounted for by a similar diffuse, uptake-sensitive mechanism.

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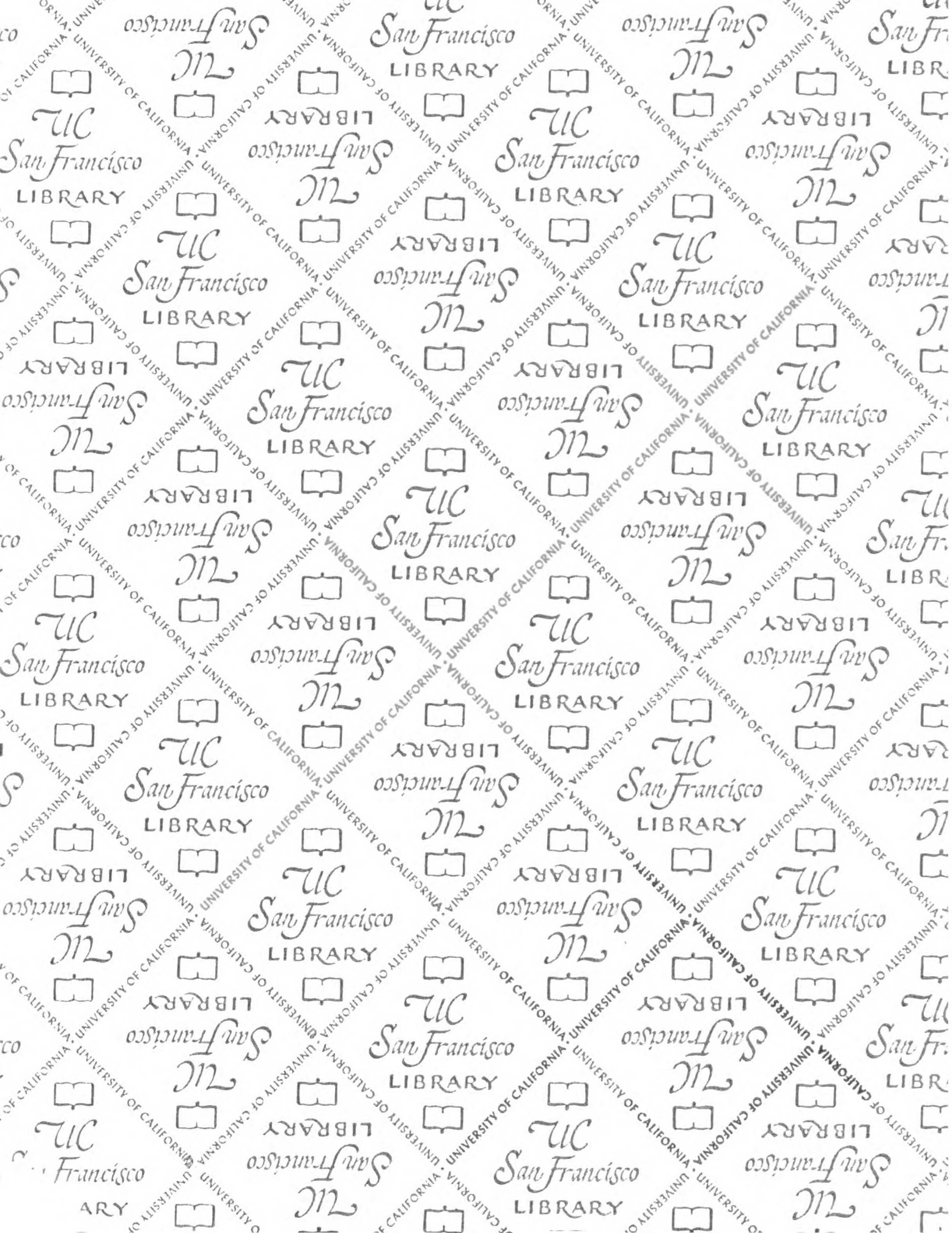
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