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GABAergic Inhibition Regulates the Synaptic Activation of Cholinergic-Dependent Plateau Potentials

by

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Abstract

The cholinergic-dependent plateau potential (PP) is a prolonged depolarization of hippocampal CA1 pyramidal neurons. The PP can be generated synaptically in CA1 pyramidal neurons of the hippocampal slice following stimulation of the Schaffer collaterals. To reliably activate the PP synaptically both $GABA_A$ and $GABA_B$ receptors need to be blocked in the presence of a muscarinic receptor agonist. The intrinsically and synaptically evoked PP appear to be generated by the same mechanism. Intracellular BAPTA perfusion prevents the generation of the PP and an increase in conductance is seen during the onset of the synaptically activated PP. In contrast, blocking L-type calcium channels prevents the intrinsic but not the synaptic activation of the PP. Blocking NMDA receptor channels as a source of calcium entry has no effect on the synaptic generation of the plateau potential.

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Hypothesis and Objectives

In the hippocampal slice preparation, applying the cholinergic agonist carbachol and evoking a burst of action potentials elicits a prolonged membrane depolarization: The cholinergic-dependent plateau potential. The hypothesis of this dissertation was:

Hypothesis:

The cholinergic-dependent plateau potential can be evoked synaptically in CA1 pyramidal cells of the rat hippocampus.

Preliminary experiments demonstrated that applying carbachol and evoking an excitatory post-synaptic response activated the cholinergic –dependent plateau potential in CA1 pyramidal neurons but this effect was inconsistent in its appearance. The objectives of this dissertation were:

Objectives:

1. To determine if inhibition played a role in preventing the consistent activation of the plateau potential.
2. To determine whether the synaptically evoked and previously described intrinsically evoked plateau potential were the same phenomena.
3. To test the dependence of the synaptically evoked plateau potential on an increase in intracellular calcium and the role played by influx through voltage and NMDA-receptor channels.

Chapter 1

Introduction

The Plateau Potential:

Previous work by Fraser and MacVicar (1996a) established that a prolonged depolarization of the membrane potential, or plateau potential, could be evoked in CA1 pyramidal cells of the rat hippocampus. This plateau potential was shown to depend on either muscarinic or metabotropic glutamate receptor stimulation and depolarization of the membrane potential with positive DC current injection to elicit spike bursting (Fraser and MacVicar,1996a; Fraser,1997). The plateau potential appears to be generated following the activation of a non-specific cation conductance and it is dependent on an increase in the intracellular calcium concentration, brought about by an influx of calcium from the extracellular environment (Fraser and MacVicar,1996a). In addition to its dependence on calcium influx, the cholinergic-dependent plateau potential requires dephosphorylation, mediated by phosphatase 1/2B (Fraser and MacVicar,1996b). An example of the plateau potential, evoked with synaptic stimulation, is shown in Figure 3A.

Seizure activity and the hippocampus:

The plateau potential is intriguing, both as a phenomena recorded in single cells of a brain slice preparation and as a potential mechanism underlying the ictal depolarizations recorded during epileptic seizure discharge. The ability to record the plateau potential in the

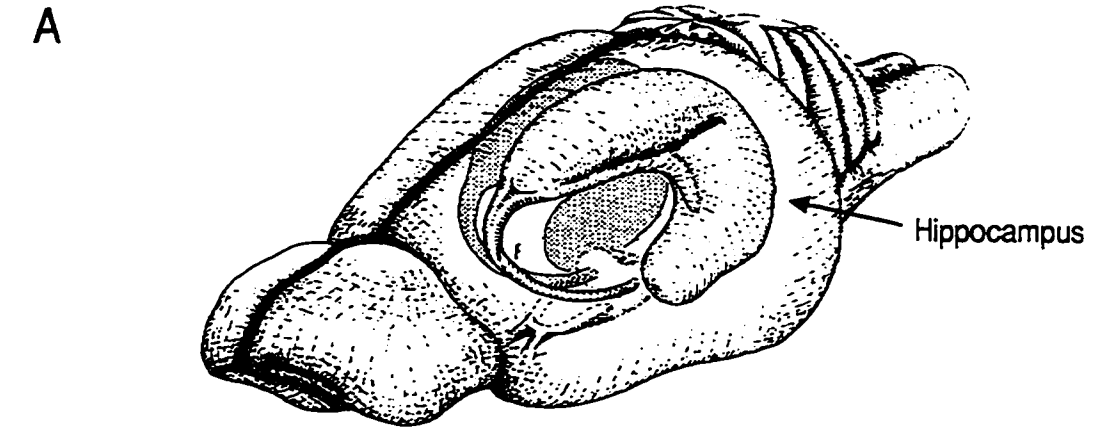
hippocampus is particularly exciting as this is a region of the brain that is susceptible to developing the abnormal, excessive discharge patterns that define seizure activity (Lothman et al.,1991; McNamara,1994). The temporal lobe, which includes the hippocampus, is the locus for the majority of complex partial seizures. These epileptic events cause an alteration in consciousness and they originate from an identifiable focal cortical site (McNamara,1994). These complex partial seizures comprise the majority of drug-resistant epilepsies whose usual treatment is surgical intervention. (Lothman et al.,1991; McNamara,1994). The seizure prone hippocampus is usually targeted and its surgical resection virtually eliminates epileptic seizures in 80-90% of the patients who are unresponsive to drug therapy (reviewed by McNamara,1994). Because the hippocampus is implicated as a critical region for generating seizure activity, much of the research into the cellular mechanisms of epilepsy has focused on this area of the brain.

Burst discharge in the hippocampus:

A number of *in vivo* and *in vitro* models for generating epileptiform discharge in the hippocampus have been developed. These approaches can be roughly grouped into four categories; the application of agents that interfere with inhibition (i.e. penicillin, bicuculline), the use of compounds that cause neuronal excitation (i.e. high potassium, low magnesium, NMDA, cholinergic agents), introduction of mechanical insults (i.e. freezing), and application of focal electrical stimulation (reviewed in Lothman et al,1991). Repeated focal electrical stimulation of the hippocampus using in-dwelling electrodes to kindle a specific brain region, has been used to effectively bring about a lasting predisposition to epileptiform convulsions (Goddard et al.,1986). A variation of the kindling protocol,

developed by Eric Lothman, utilizes a distinct pattern of repeated electrical stimulation of the rat hippocampus to bring about a period of self-sustaining limbic status epilepticus (SSLSE) that continues for 6-18 hours post-stimulus (Mangan and Lothman,1996). This differs from the traditional kindling model where seizure activity is only seen following a repetition of the original kindling stimulus and the convulsions do not sustain themselves when the stimulation is terminated. The successful induction of seizure-like activity can be determined in a number of ways. A change in the behavior of the treated animal can be observed (i.e. when complex partial seizures of a given severity are reached), characteristic interictal (between seizure) and ictal (seizure-like) discharge patterns can be recorded using surface EEG, or seizure-like electrical activity can be recorded in a population of cells or individual neurons of a particular brain region. Surface EEG is commonly used to record the characteristic interictal burst, or spike pattern, that is used to confirm the diagnosis of epilepsy *in vivo* (Sperling,1988). The sharp wave and spike pattern seen on the EEG, depolarizations lasting less than a second each, arise from the synchronous depolarization of a pool of neurons (Sperling,1988). Using interictal spikes on the EEG trace as a definitive indicator of seizure activity and the use of EEG recordings to pinpoint seizure loci are both questionable. Spikes can be found on the EEGs of individuals asymptomatic for epileptic seizure and surface EEG recordings do not locate the seizure locus in the majority of patients who undergo successful surgical resection procedures (Sperling,1988; Rayport et al.,1986). Some of the shortcomings of surface recordings include the difficulty to record activity in deeper tissue layers and the inconsistent results obtained when EEG records are used to identify epileptic foci. To overcome some of these problems implanted intracranial electrodes have been used, both in human patients and animal models (Sperling,1988; Rayport et al.,1986). Depending on the placement of the electrodes, the

initiation and propagation of ictal, or seizure related, activity can be monitored in different brain regions. These types of recordings have confirmed that ictal bursting activity, sustained depolarizations that often last tens of seconds, originates primarily within the hippocampus or amygdala and rapidly propagates between these two areas (Buser et al.,1971; Sperling,1988). In order to resolve seizure related events within the hippocampus itself, the slice preparation has been used to provide an *in vitro* model that preserves many of the intra-hippocampal synaptic connections that contribute to the activity recorded in the intact brain (see Figure 1). By using the slice preparation, simultaneous intracellular and field potential recordings can be made in separate hippocampal regions during the induction of seizure activity or following the induction of seizure activity in the whole animal (Traynelis and Dingledine,1988; Mangan and Lothman,1996; Esclapez et al.,1997). A combined entorhinal cortex- hippocampal slice has been developed that allows for the full tri-synaptic hippocampal circuit to be kept intact *in vitro* (Nagao et al.,1996; Rafiq et al.,1993). When this combined slice is stimulated with the muscarinic agonist pilocarpine, epileptiform activity is induced (Nagao et al.,1996). Simultaneous field recordings show that pilocarpine induced ictal bursting originates in the entorhinal cortex and then propagates through the perforant pathway to the dentate gyrus (Nagao et al.,1996). From the dentate gyrus, epileptiform bursting propagates to the CA3 and CA1 fields (Nagao et al.,1996). When the hippocampus proper is isolated from the entorhinal cortex in a slice model, epileptiform activity can still be evoked. The slices can be "made epileptic" by kindling the animal that the slices are prepared from, administering high potassium aCSF to the slice, or applying cholinergic agonists (Somjen et al.,1985; Traynelis and Dingledine,1988; Williams and Kauer,1997). The CA1 region generates ictal bursts in these seizure prone slices, prolonged depolarizations that aren't seen in the dentate gyrus or



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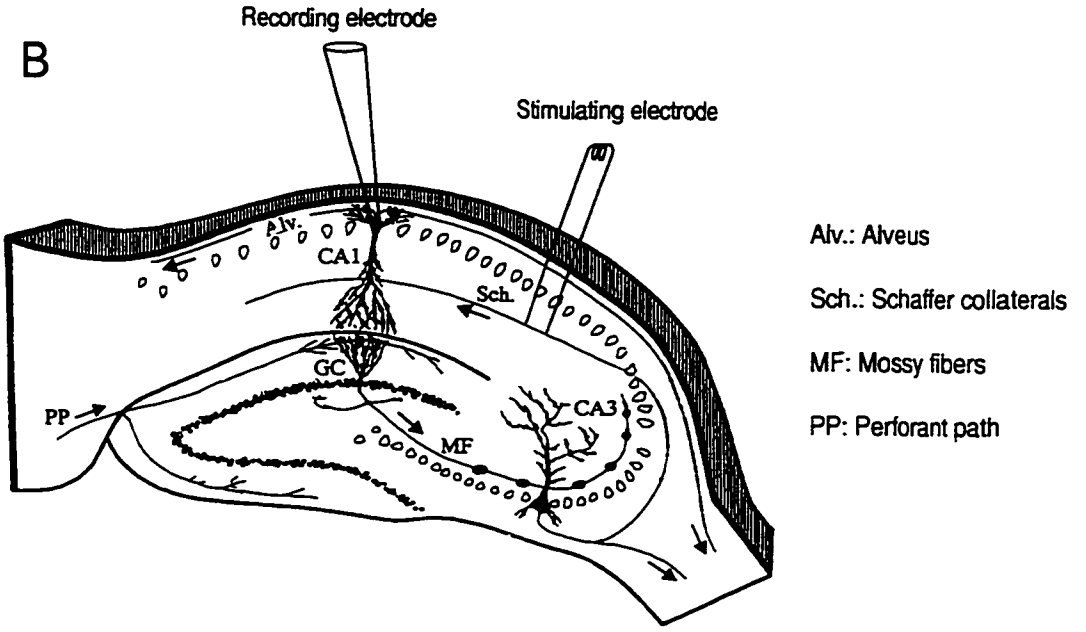

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Figure 1

A. The intact hippocampal formation and its location within the rat brain.

B. A representation of the transverse hippocampal slice and the tri-synaptic pathway. Approximate position of the stimulating and recording electrodes is shown.

CA3 region (Traynelis and Dingledine,1988; Williams and Kauer,1997). The ictal bursts in area CA1 are clearly driven by rhythmic activity within the CA3 neuronal population and rely on intact synaptic connections between CA3 and CA1 to be generated (Traynelis and Dingledine,1988; Williams and Kauer,1997).

Directly correlating EEG or depth electrode recordings of seizure activity in human patients or animals induced to be epileptic with single cell recordings of prolonged depolarization in the hippocampus is not possible at this time. By combining information from the recordings of epileptic activity that have been made, from the intact brain to the slice preparation, I hope to be able to make a link between the cholinergic dependent plateau potential and ictal discharge during epileptic seizure.

The ictal depolarization that is seen with surface or indwelling electrodes in human patients is distinguished as a prolonged depolarization arising within a population of neurons (Sperling,1988; Rayport et al.,1986). In complex, partial seizures the hippocampus has been implicated as a generator of epileptic activity (Lothman et al.,1991; McNamara,1994). Depth electrodes in human patients help confirm that ictal discharge patterns originate within the hippocampus in a majority of patients (Rayport et al.,1986). *In vitro* studies using the hippocampal slice preparation correlate field activity during evoked epileptiform bursting, similar to what is seen with depth electrodes *in vivo*, with prolonged depolarizations of individual CA1 neurons (Somjen et al.,1985). This ictal-like activity in CA1 neurons has been shown to be synaptically driven by population activity within the CA3 region (Traynelis and Dingledine,1988; Williams and Kauer,1997). The cholinergic-dependent plateau potential has been recorded in individual pyramidal cells of the rat

hippocampal CA1 region using whole cell patch recordings (Fraser and MacVicar,1996a). The waveform of the cholinergic-dependent plateau potential bears a resemblance in duration and activation/decay kinetics to the synaptically driven ictal bursts within the CA1 region. If the plateau potential can be evoked synaptically in the hippocampal slice model, its potential role as a basic mechanism underlying ictal bursting could be further established.

Cholinergic input to the hippocampus:

The plateau potential evoked in CA1 pyramidal neurons is dependent on cholinergic stimulation acting through muscarinic receptors (Fraser and MacVicar,1996a). To plausibly establish a role for the plateau potential in the pathophysiology of epileptic seizure activity, the presence of substantial cholinergic input to the hippocampus is essential.

Work on the specific connections contained within the septo-hippocampal transmission pathway has established that neurons within the hippocampal CA1 region receive afferent projections from the septal area (see Figure 2A). Two types of fibers can be distinguished within this septo-hippocampal projection, thicker type I fibers that innervate outside of the pyramidal cell layer and thinner type II fibers that project heavily to the pyramidal layer (Amaral and Witter,1995; Nyakas et al.,1987; Frotscher and Leranth,1985). These projections originate within the medial septum and the vertical limb of the diagonal band of Broca (VDB), collectively referred to as the MSDB (Nyakas et al.,1987). The input from the VDB is the most substantial (Nyakas et al.,1987). The type I fibers project from

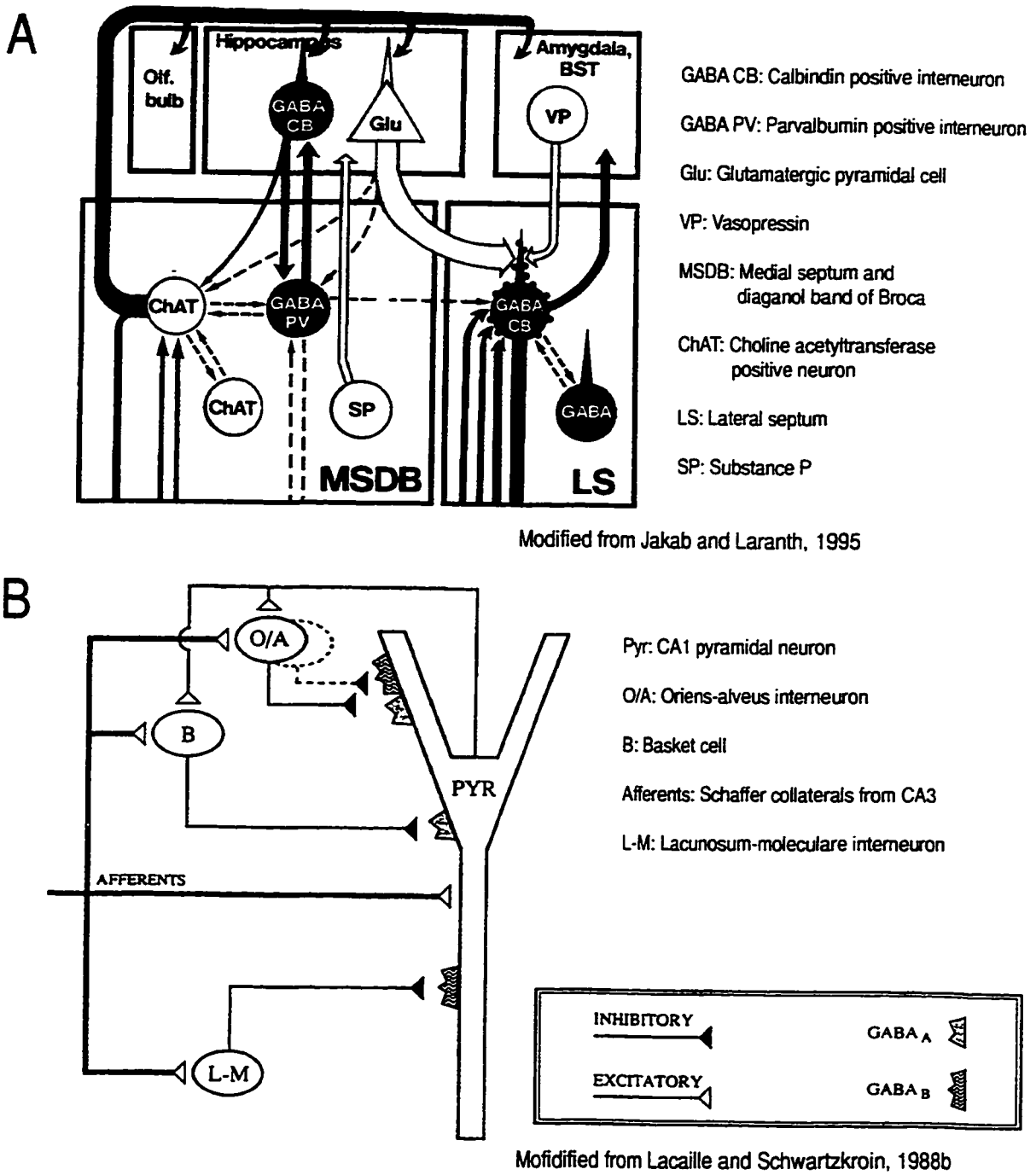

Figure 2

A. Extensive connections are formed between the septum and the hippocampus. GABAergic neurons in the septum and hippocampus form reciprocal synaptic connections. Cholinergic neurons of the septum synapse onto pyramidal cells of the CA1 hippocampal region.

B. Three subtypes of CA1 interneuron and pyramidal cells receive excitatory input from CA3 afferents. These interneurons form inhibitory, GABA mediated synaptic connections with CA1 pyramidal cells. Both O/A and basket interneurons receive excitatory input from CA1 pyramidal cell afferents.

GABAergic septal neurons and form synaptic contacts with calbindin containing CA1 interneurons (Nyakas et al.,1987; Frotscher and Leranth,1985) while the type II fibers synaptically target the soma and proximal dendrites of pyramidal neurons in the CA1 region, releasing acetylcholine at their presynaptic terminus (Nyakas et al.,1987; Frotscher and Leranth,1985). GABAergic interneurons within the hippocampus in turn project back on to parvalbumin containing, GABAergic neurons within the MSDB, forming a synaptic loop between these two areas (Amaral and Witter,1995). The type I GABAergic afferents from the septum have been shown to disinhibit hippocampal pyramidal cells by producing IPSPs in hippocampal interneurons (Freund and Antal,1988; Toth et al.,1997).

Cholinergic receptors:

The acetylcholine that is released in the CA1 region of the hippocampus can have a number of effects on the pyramidal cells and interneurons found there. While α-bungarotoxin-sensitive nicotinic acetylcholine receptors are present on CA1 interneurons only, all five cloned subtypes of muscarinic receptor are expressed throughout the pyramidal cell body layer of the CA1 region (Frazier et al.,1998; Levey et al.,1995; Vilaro et al.,1993). The five subtypes of muscarinic receptor all functionally couple to G-proteins and can be divided into two general categories based on the selectivity of their G-protein interaction. The odd numbered receptor subtypes, m1, m3, and m5, couple with a pertussis toxin insensitive G-protein ($G_{q/11}$) to stimulate phospholipase C (reviewed by Brann et al.,1993). This leads to the liberation of inositol 1,4,5-triphosphate and diacylglycerol with a subsequent mobilization of calcium from intracellular stores (Felder,1995). The even numbered

receptor subtypes, m2 and m4, act through the pertussis toxin sensitive $G_{i/o}$ to inhibit adenylate cyclase and its subsequent formation of cAMP (Felder,1995). By binding to muscarinic receptors, acetylcholine increases the excitability of CA1 pyramidal cells through an inhibition of a number of potassium conductances; including those underlying the calcium dependent I_{AHP}, the voltage-gated I_M, and a time-independent leak potassium conductance (Brown et al.,1989; Cole and Nicoll,1984; Madison et al.,1987). Activation of muscarinic receptors following systemic administration of the muscarinic agonist pilocarpine can lead to a persistent and inappropriate activation of hippocampal neurons that leads to seizure like bursting that spreads from the hippocampus to other cortical regions (Esclapez,1997; Turski et al.,1984). More specifically, the m1 muscarinic receptor seems to play a key role in the induction of seizure that is brought about with pilocarpine injection. Transgenic mice with an m1 muscarinic receptor knockout are resistant to pilocarpine induced seizures but remain susceptible to kainate induced seizures (Hamilton et al., 1997).

The hippocampus:

While the septum provides significant input to the pyramidal cells and interneurons of the hippocampal CA1 region, the afferent fibers of the hippocampal circuit provide the main conduit for synaptic transmission in this brain structure and eventually provide the CA1 region with its primary source of excitatory input (Amaral and Witter, 1995). The hippocampus can be separated into generalized regions that contain characteristic collections of neurons and form distinct, largely unidirectional synaptic projections to other hippocampal regions (reviewed by Amaral and Witter, 1995).

The tri-synaptic hippocampal circuit begins in the entorhinal cortex, from where afferent fibers project along the perforant path to the dentate gyrus (Amaral and Witter, 1995). The granule cells of the dentate gyrus that receive input from the entorhinal cortex, then project, via the mossy fibers, to the CA3 field within the hippocampus proper. Pyramidal cells in the CA3 region form extensive projections within their own neuronal population. This allows for polysynaptic, recurrent excitation to occur in this area (Lebovitz et al.,1971; MacVicar and Tse,1989). These extensive local circuits underlie the synchronous activity that is seen in the CA3 region when the cholinergic agonist carbachol is applied to the hippocampal slice preparation (MacVicar and Tse,1989; Williams and Kauer,1997). The rhythmical, synchronous activity that cholinergic agonist application induces in the CA3 region drives a closely matched rhythmical bursting activity in the CA1 region that intermittently evolves into a longer lasting depolarization in CA1 but not CA3 (Williams and Kauer,1997). This repetitive depolarization of the membrane potential of both CA3 and CA1 pyramidal cells appears to originate in the CA3 region as its appearance can be blocked in CA1 but not CA3 by making a knife cut through the fibers that contain the CA3 efferents.

The fiber tract that passes from the CA3 to the CA1 region, the Schaffer collaterals, provides synaptic output from CA3 neurons to pyramidal cells and interneurons of the CA1 region (Amaral and Witter, 1995). The CA3 pyramidal cell efferents contained in the Schaffer collaterals are primarily glutamatergic. The pyramidal cells of the CA1 region contain a number of glutamate receptor subtypes that allow them to respond in a variety of ways to this glutamatergic input.

Ionotropic glutamate receptors of both the NMDA and AMPA subtype have been functionally localized to both the soma and apical dendrites of CA1 pyramidal neurons (Spruston et al.,1995). These receptors mediate excitatory post-synaptic currents in CA1 pyramidal cells at their resting membrane potential of -64 mV (Spruston and Johnston,1992; Spruston et al.,1995). CA1 pyramidal neuron AMPA receptor channels are relatively impermeable to calcium and the charge they carry when activated at the resting membrane potential is primarily due to influx of sodium ions (Jonas and Sakmann, 1992; Spruston et al.,1995). NMDA receptor channels, once relieved of their Mg^{2+} block by membrane depolarization, carry charge through the influx of both sodium and calcium ions (Spruston et al.,1995). In addition to these ionotropic receptors, metabotropic glutamate receptors (mGluRs) are expressed in the hippocampus. Of the various mGluR subtypes, mGluR5, a member of the group I mGluR subfamily, is expressed at the highest level in the CA1 region (Fotuhi,1994). Like the m1 muscarinic receptor, mGluR5 is coupled to the stimulation of phosphatidylinositol hydrolysis and subsequent intracellular calcium mediated signal transduction (Abe et al.,1992).

Because of their permeability to calcium, NMDA receptor channels can serve as a significant source of somatic and dendritic calcium entry in CA1 pyramidal cells during excitatory synaptic events. Additionally, acetylcholine has been shown to potentiate NMDA-evoked calcium influx in hippocampal neurons through an atropine sensitive, m1 muscarinic receptor mediated mechanism, possibly through the release of calcium from intracellular stores (Segal, 1992; Hashiguchi et al.,1997).

Another major source of activity dependent calcium influx in CA1 pyramidal cells is through voltage gated calcium channels. L-type high voltage activated (HVA) calcium channels are located on the cell bodies and proximal apical dendrites of CA1 pyramidal cells (Westenbroek et al., 1990). N-type HVA Ca^{2+} channels are found both on the somata and over the full length of the dendritic arbour of CA1 neurons, while HVA R and low voltage activated (LVA) T type channels are found in abundance on the distal apical dendrites. (Mills et al., 1994, Magee and Johnston,1995b). Voltage gated calcium channels also contribute to presynaptic release at the CA3-CA1 synapse. N and Q-type calcium channels play a large role in presynaptic calcium entry and subsequent transmitter release from CA3 axon terminals (Wu and Saggau, 1994; Wheeler et al., 1994).

Organization of the CA1 region:

Within the CA1 field of the hippocampus proper, a number of neuron types are arranged in distinct layers where they form an interconnected network of synaptic connections. The principal output cell of the CA1 region is the pyramidal neuron whose numbers have been estimated to be approximately 420,000-750,000 (Amaral et al., 1990; reviewed by Thompson, 1994). The cell bodies of these pyramidal neurons are organized within the stratum pyramidale in an anatomically identifiable layer and, in combination with their dendrites that extend into the stratum radiatum apically and stratum oriens basally, receive 5000-10,000 excitatory synapses each (Amaral et al., 1990). Three distinct types of interneurons have been identified within different layers of the CA1 region (see Figure 2B). Basket cells are found at the stratum pyramidale/oriens border while the cell bodies of oriens/alveus (O/A) interneurons are located in the stratum oriens- alveus border and L-M

interneurons are found at the border between stratum lacunosum- and stratum radiatum (Thompson, 1994). These interneurons are principally GABAergic and many contain peptides like somatastatin and CCH (Thompson, 1994). The major role played by the interneurons in the CA1 region is to regulate, through inhibitory GABAergic synaptic transmission, the excitability of the pyramidal cells within their local region (Thompson, 1994). These interneurons form synapses onto CA1 pyramidal cell bodies and dendrites that strategically allow them to prevent continuous firing of the pyramidal cells in response to the extensive excitatory input from the CA3 region, even though the pyramidal cells outnumber the interneurons by one or two orders of magnitude (reviewed by Thompson, 1994). Basket cells are so named because of the shape of the axonal plexus that they form around the pyramidal cell somata. Basket cells make efferent synaptic connections with pyramidal cells, primarily on the somata but also on proximal dendrites (Buhl et al.,1995). It is believed that the primary receptor type located on the pyramidal cell post-synaptic site of the basket cell-pyramidal cell synapse is of the $GABA_A$ variety (Buhl et al.,1995). O/A interneurons also form extensive efferent projections to CA1 pyramidal cells, forming synapses on both the proximal basal dendrites and more importantly on the axon initial segment, an area that plays a critical role in action potential generation (Lacaille et al.,1987). O/A interneurons and basket cells receive afferent input from both the Schaffer collateral CA3-CA1 synapse, mediating feed forward inhibition of the pyramidal cell population through activation of these interneuron populations, and from CA1 pyramidal cell axons, which mediate feedback inhibition of the pyramidal cell population through activation of these interneurons (Lacaille et al.,1987; Lacaille et al.,1989). In contrast the L-M interneurons only receive excitatory afferent input from CA3 pyramidal cell axons and take part in feedforward inhibition only (Lacaille and Schwartzkroin,1988b). The L-M

interneurons form synaptic connections with the apical dendrites of the pyramidal cells, where the primary postsynaptic receptor is thought to be of the $GABA_B$ subtype (Lacaille and Schwartzkroin,1988b). By utilizing both feed-forward and feedback inhibition and through the strategic placement of synaptic connections on the dendrites, soma, and axon initial segment of pyramidal cells, the interneurons of the CA1 region are able to effectively counter neuronal excitation and restrain excessive neural activity within the local cell population. During stimulation of the Schaffer collaterals in the hippocampal slice, both feedforward and feedback inhibition are activated through stimulation of the pyramidal cell and interneuron population (Lacaille et al.,1987; Lacaille et al.,1989; Lacaille and Schwartzkroin,1988b).

GABA and epilepsy:

Many of the manipulations that have been used to experimentally generate epileptic-like seizure activity in animal models involve application of antagonists of GABAergic synaptic transmission. $GABA_A$ receptor antagonists like bicuculline and picrotoxin have been used for over 30 years to induce epileptic-like discharge in whole animals and brain slice preparations (reviewed in Thompson,1994). Additionally some of the animal models that have been developed to simulate aspects of human temporal lobe epilepsy display a pronounced reduction in GABAergic neurotransmission throughout the hippocampus following induction of seizure activity (reviewed by Bradford,1995). This disruption in the balance between inhibition and excitation does not appear to be a result of hippocampal interneuron loss in tissue from epileptic patients (Babb et al,1989). In addition to the importance of $GABA_A$ receptor activity in many models of seizure discharge, changes in

$GABA_B$ receptor properties may also appear in association with the onset of epileptic seizure activity. The role that $GABA_B$ receptors might be playing in seizure activity has been demonstrated in the SSLSE model of seizure induction. Following induction of SSLSE, the IPSP recorded in pyramidal cells within the hippocampal slice preparation is of similar amplitude but is shorter in duration than the IPSP seen in control tissue. The long lasting component of the IPSP ($GABA_B$ receptor mediated) that is present in control tissue is not seen post-SSLSE and the quickly activating/inactivating response that remains is completely abolished by picrotoxin. It appears that $GABA_B$ receptors at both the presynaptic and postsynaptic site are affected in this animal model. A presynaptic inhibition of GABA release through a $GABA_B$ autoreceptor is seen in control tissue. This is tested by applying the $GABA_B$ agonist baclofen to the slice preparation and looking to see if there is any reduction in IPSP amplitude. In contrast to control tissue, baclofen doesn't reduce the IPSP amplitude in SSLSE tissue. CGP 55845A, a $GABA_B$ antagonist, doesn't cause the reduction in the late component of the IPSP in the SSLSE slices that is seen in control slices. Simply blocking hippocampal $GABA_A$ receptors to simulate epileptic activity may not provide a complete picture of the events that underlie the sustained depolarizations that typify seizure discharge. Bicuculline has been shown to enhance the late, phaclofen sensitive component of inhibitory synaptic transmission in the hippocampus, the $GABA_B$ component of the IPSP (Stanford et al., 1995). Blocking both $GABA_A$ and $GABA_B$ receptors might be necessary to reveal the sustained pyramidal cell depolarizations that truly underlie ictal bursting. A reduction in both the $GABA_A$ and $GABA_B$ receptor mediated component of inhibitory synaptic transmission might be important in the development of epileptic seizure activity.

Changes in GABA inhibition that often accompany the process of seizure induction might give us some clues regarding the changes that are occurring at the cellular level when various manipulations that produce seizure discharge in the hippocampus are utilized. The cholinergic-dependent plateau potential, a candidate cellular correlate for ictal depolarization in the hippocampus, shows a strong dependence on muscarinic receptor stimulation and calcium influx for its genesis (Fraser and MacVicar,1996a). Recent work in CA1 pyramidal neuron dendritic physiology may help to make a connection between the changes in GABAergic inhibition that are seen in models of seizure induction, the characteristics of the cholinergic-dependent plateau potential, and basic mechanisms of burst discharge operating at the level of the individual hippocampal neuron.

Dendritic properties:

The plateau potential is dependent on calcium influx in CA1 pyramidal neurons (Fraser and MacVicar,1996a). The distribution of voltage gated calcium channels along the somto-dendritic axis and any active dendritic processes that amplify calcium entry through these channels will probably be critical to the synaptic generation of the plateau potential. Voltage-gated sodium channel dependent action potentials initiated in the soma (or the dendrites following suprathreshold synaptic stimulation) of CA1 pyramidal cells can be actively propagated by dendritic ion conductances from the soma of the pyramidal cell out to the distal portions of the dendritic tree (Turner et al.,1991; Jaffe et al.,1992; Spruston et al.,1995). This active propagation of action potentials into the dendritic arborization results in an increase in intracellular calcium and sodium concentration by opening voltage-gated channels to these ions (Jaffe et al.,1992; Magee and Johnston,1995a). Calcium spikes can

be initiated in the distal, apical dendrites of CA1 pyramidal cells, with a lower threshold of injected positive current required to generate calcium spike firing in the dendrites than is required in the soma (Golding and Spruston, 1997). Action potentials back-propagating through the apical dendrites of CA1 pyramidal cells undergo activity dependent attenuation (Spruston et al.,1995). This results in a reduction in the amplitude of successive back propagated dendritic spikes during a train of action potentials. The attenuation of dendritic spikes is greater as the distance from the soma increases. The amplitude of the last spike compared with the first spike in a train of evoked action potentials shows a slight, approximately 20%, decrease at the soma. In the distal dendrites, 300 μm from the soma, a decrease of approximately 80% is seen. It has been shown that this decrease in spike amplitude can be reversed by the application of carbachol, acting through an atropine sensitive mechanism (Tsubokawa and Ross,1997). The activity dependent reduction first reported by Spruston (Spruston et al.,1995) acts in addition to the distance dependent reduction in initial action potential amplitude. The amplitude of the initial or single back-propagated dendritic action potential decreases with increasing distance from the cell body (Turner et al.,1991; Jaffe et al.,1992; Spruston et al.,1995). Inhibitory input to the CA1 pyramidal cell dendrites can further modulate this distance dependent reduction in dendritically propagated action potential amplitude. Pairing inhibitory input to the apical dendrites with intrinsically stimulated action potential firing in the soma of CA1 pyramidal cells causes a reduction in initial or single action potential amplitude and intracellular calcium increase measured 300 μm from the soma in the apical dendrites (Tsubokawa and Ross,1996; Kamondi et al.,1997). This reduction in intracellular calcium concentration increase, action potential amplitude and the appearance of synaptically evoked calcium

spikes in the distal dendrites is reversed following the application of the $GABA_A$ receptor antagonist bicuculline methiodide (Tsubokawa and Ross, 1996; Golding and Spruston, 1997).

Methods and Materials:

Hippocampal Slice Preparation

Sprague-Dawley rats, post-natal 15-21 days, were anaesthetized with ether and decapitated. The extracted brain was immersed in chilled artificial cerebrospinal fluid (aCSF, see Table 1 for composition) and a block of tissue containing the hippocampus was cut with a razor blade. The anterior surface of the blocked tissue was then attached to the stage of a vibrating micro-slicer with cyanoacrylate glue. Stability was enhanced by propping the dorsal surface of the blocked brain against an immobilized block of 2% agar. Transverse slices (400-450 μm) were prepared by sectioning the hippocampus perpendicular to its septotemporal axis. The cortex and midbrain were dissected away in a dish containing bubbled aCSF and the isolated hippocampal slices were transferred to an interface chamber at room temperature.

Whole-Cell Patch Clamp Recording

Whole-cell current clamp recordings from neurons within hippocampal slices were obtained using the "blind-patch" technique (Blanton et al., 1989). Slices were individually transferred to a recording chamber located on a dissecting microscope and submerged in rapidly flowing (1ml/min) oxygenated aCSF. Bath temperature was maintained at 34-35°C with a Peltier unit and Cambion bipolar controller. Patch-electrodes of 5-7 MΩ were pulled on either a Narishige PP-83 or a Sutter Instruments P-97 microelectrode puller from 1.5

outer diameter thin walled glass (150F-4, World Precision Instruments) and filled with intracellular solution (see Table 1 for composition). Once a gigaohm seal was established within stratum pyramidale, whole cell access was gained at an electrode potential of -65mV. This electrode potential was chosen to approximate the normal resting potential of CA1 pyramidal neurons (Spruston and Johnston,1992). Voltage recordings were obtained in bridge mode (Axoclamp-2A; Axon Instruments) and were lowpass filtered (4-pole Bessel) at 10 kHz (-3 dB). Capacitance neutralization was fully maximized and the voltage drop across the electrode patch subtracted by bridge circuit potentiometer. Data was digitized with a Tl-1 A/D interface and acquired using pCLAMP computer software (Axon Instruments). Series resistance was determined via a bridge potentiometer by balancing the voltage drop across the patch in response to a negative current pulse (-30 pA; 10 msec) and recordings with series resistance greater than 20 MΩ were discarded. Pyramidal neurons recorded in whole cell mode were stimulated in two ways. Membrane depolarization was generated through the application of positive current steps through the recording electrode (intrinsic stimulation) or stimulation trains delivered through a bipolar, teflon coated nickel stimulating electrode placed in stratum radiatum at the CA2-CA1 border (synaptic stimulation). Stimulation was delivered for 200 ms at 65 Hz in all of the experiments in which GABA antagonists, alone or in the presence of oxotremorine M, were applied. The intensity of the stimulation was set between 20 - 40 mV during these episodes of synaptic stimulation. During experiments in which only a cholinergic (carbachol) or muscarinic (oxotremorine M) agonist were applied, stimulation was delivered for 800 ms at up to 100 Hz at intensities ranging from 20 to 60 mV.

Solution	Concentration of constituent in mM
aCSF	126 NaCl 2.5 KCl 2.0 $MgCl_2$ 1.25 NaH_2PO_4 26 $NaHCO_3$ 10 Glucose 2 $CaCl_2$ (added from a 1M aqueous stock after bubbling solution containing other elements with 5% CO_2 / 95% O_2 for >15 minutes) pH = 7.4
Intracellular solution	140 K-gluconate 1.1 EGTA 0.1 $CaCl_2$ 10 Hepes 2 Mg-ATP 0.3 Na-GTP pH=7.2
BAPTA intracellular solution	100 K-gluconate 40 Hepes 10 K-BAPTA 2 Mg-ATP 0.3 Na-GTP pH=7.2
Oxotremorine M, atropine, and CGP 35348	Stored as a 10mM aqueous stock in aliquots at –20°C. Thawed and added to aCSF on day of use.
Bicuculline methiodide	Stored as a 25mM aqueous stock in aliquots at –20°C. Thawed and added to aCSF on day of use.
Nimodipine	Stored as a 10mM stock in 95% ethanol in aliquots at –20°C. Thawed and added to aCSF on day of use.

Table 1

A listing of the extracellular solution (aCSF), intracellular solutions, and drugs applied to the extracellular bath.

Chapter 2

Results

Can the cholinergic-dependent plateau potential be evoked in CA1 pyramidal cells within the hippocampal slice preparation following the administration of cholinergic stimulation and activation of the Schaffer collaterals that provide excitatory synaptic input to the CA1 region?

Synaptically activating the plateau potential:

In the initial experiments I attempted to evoke the plateau potential by activating cholinergic receptors while applying electrical stimulation, to evoke a synaptic response in CA1 pyramidal cells, through a bipolar electrode placed in stratum radiatum of the CA2 region. Based on the description of the intrinsically evoked plateau potential provided by Fraser and MacVicar, I hypothesized that in the presence of 20μM carbachol stimulation of the Schaffer collaterals would induce sufficient post-synaptic excitation of CA1 neurons to increase intracellular calcium levels and generate the plateau potential (Fraser and MacVicar,1996a). A number of trials using this protocol were attempted before the plateau potential was synaptically activated in a CA1 pyramidal cell. Attempts to repeat this finding demonstrated that the plateau potential couldn't be consistently elicited with the application of carbachol and synaptic stimulation. In four subsequent experiments in which synaptic and intrinsic stimulation were utilized, the plateau potential was synaptically evoked twice while it was evoked with intrinsic stimulation (positive current steps) in all

four cells (see Figure 3B). It appeared that the postsynaptic response to the electrical stimulation of the Schaffer collaterals was robust even in the absence of the plateau potential, indicating that the afferent fibers were probably in good health and capable of synaptic release. Further studies were required to ascertain whether there is more to consider when synaptic, rather then intrinsic stimulation was used to generate the cholinergic-dependent plateau potential.

Pre-synaptic inhibition:

Although there appeared to be a reasonable level of post-synaptic excitation, generated by stimulation of the afferents, there may have been some forms of pre-synaptic inhibition preventing maximal synaptic release. Acetylcholine has been shown to inhibit the endogenous release of glutamate in the hippocampus by binding to muscarinic receptors of the m2 subtype, located at the pre-synaptic site (Marchi and Raiteri,1989). Because the cholinergic agonist carbachol was being applied to the whole slice, there was a concern that it may be reducing the level of glutamate released during synaptic stimulation. I then attempted to more specifically activate the post-synaptic m1 muscarinic receptor that has been shown to play a key role in plateau potential genesis (Fraser 1997) by applying oxotremorine M, a supposed m1 agonist, instead of carbachol (Birdsall et al.,1978). More recent evidence has indicated that oxotremorine M is not specific for the m1 subtype of muscarinic receptor but instead acts as an effective agonist for all classes of muscarinic receptor (Loudon et al.,1997). Not surprisingly then, the application of oxotremorine M wasn't more effective than applying carbachol in the consistent activation of the plateau

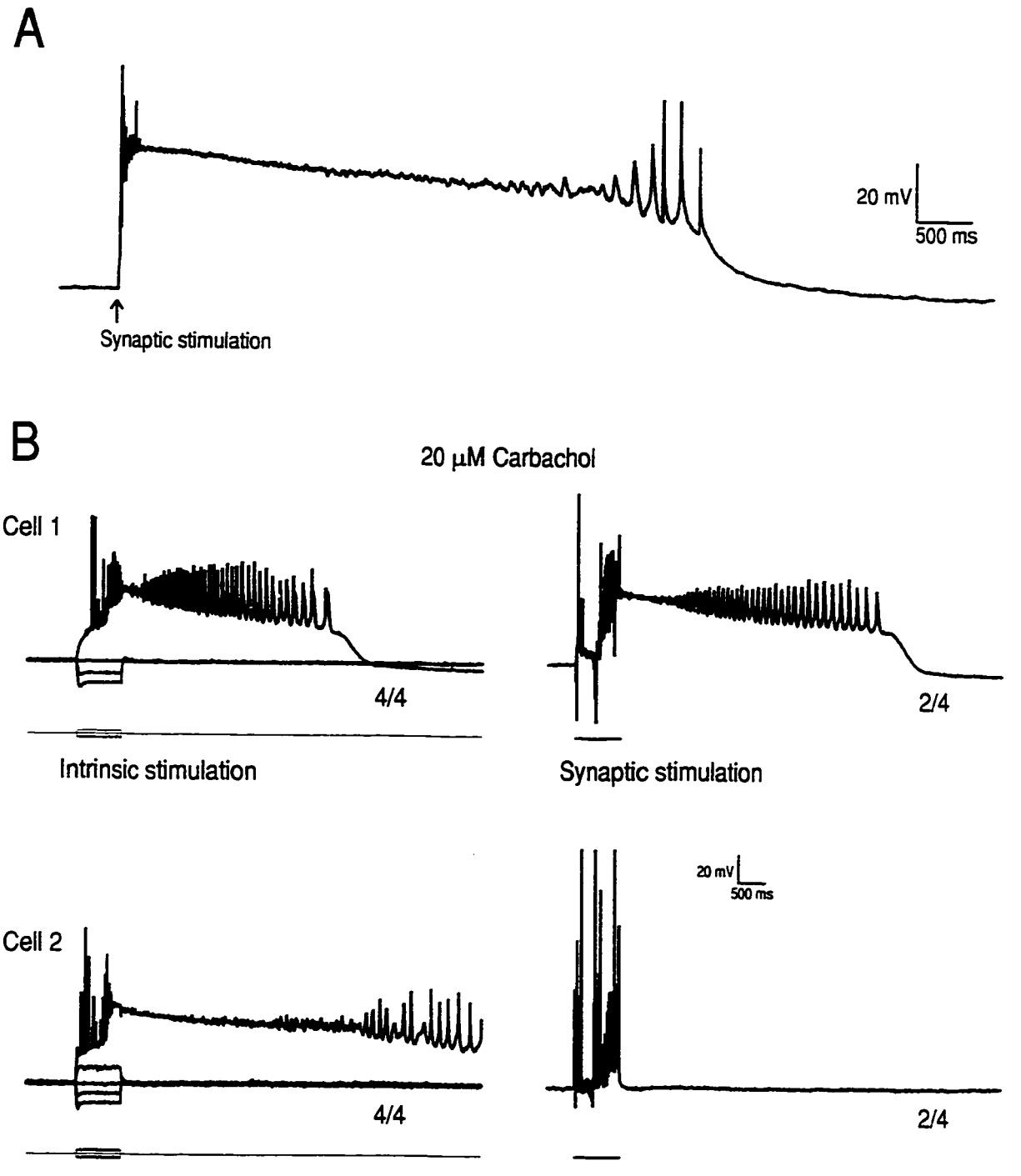

Figure 3

A. The plateau potential is a prolonged depolarization of the membrane potential. At its onset the plateau potential has a membrane potential of approximately -20 mV.

B. Application of the cholinergic agonist carbachol was sufficient for the intrinsic activation of the plateau potential in all four cells but it was only sufficient for the synaptic activation in two of the four cells tested.

potential, and no plateau potential could be evoked synaptically in the two cells tested (Figure 4A).

Adenosine has also been shown to have potent inhibitory effects on glutamatergic EPSPs in the hippocampus. (Thompson et al.,1992) Normally present at concentrations of 1μM in cerebrospinal fluid, adenosine levels are raised considerably during intense neuronal activity (Thompson et al.,1992) . Acting through DPCPX sensitive, A1 receptors in the hippocampus, adenosine depresses transmitter release pre-synaptically and hyperpolarizes postsynaptic neurons by activating a potassium conductance (Thompson et al.,1992). By synaptically stimulating fibers passing through the alveus / stratum oriens of area CA1, Morton and Davies demonstrated that significant levels of endogenous adenosine are released (Morton and Davies,1997). They assayed for the presence of endogenous adenosine by applying an adenosine kinase inhibitor to the hippocampal slice, preventing its rapid conversion to adenosine monophosphate (Morton and Davies,1997; Pak et al,1994). Both a postsynaptic hyperpolarization and a DPCPX-sensitive inhibition of a cholinergically stimulated prolonged depolarization were observed in the presence of the adenosine kinase inhibitor (Morton and Davies,1997). To determine whether adenosine release might be preventing the synaptic activation of the plateau potential in my preparation, I tested the effect of blocking A1 receptors with DPCPX. My attempts to synaptically evoke the plateau potential following the application of DPCPX or oxotremorine M and DPCPX, were unsuccessful.

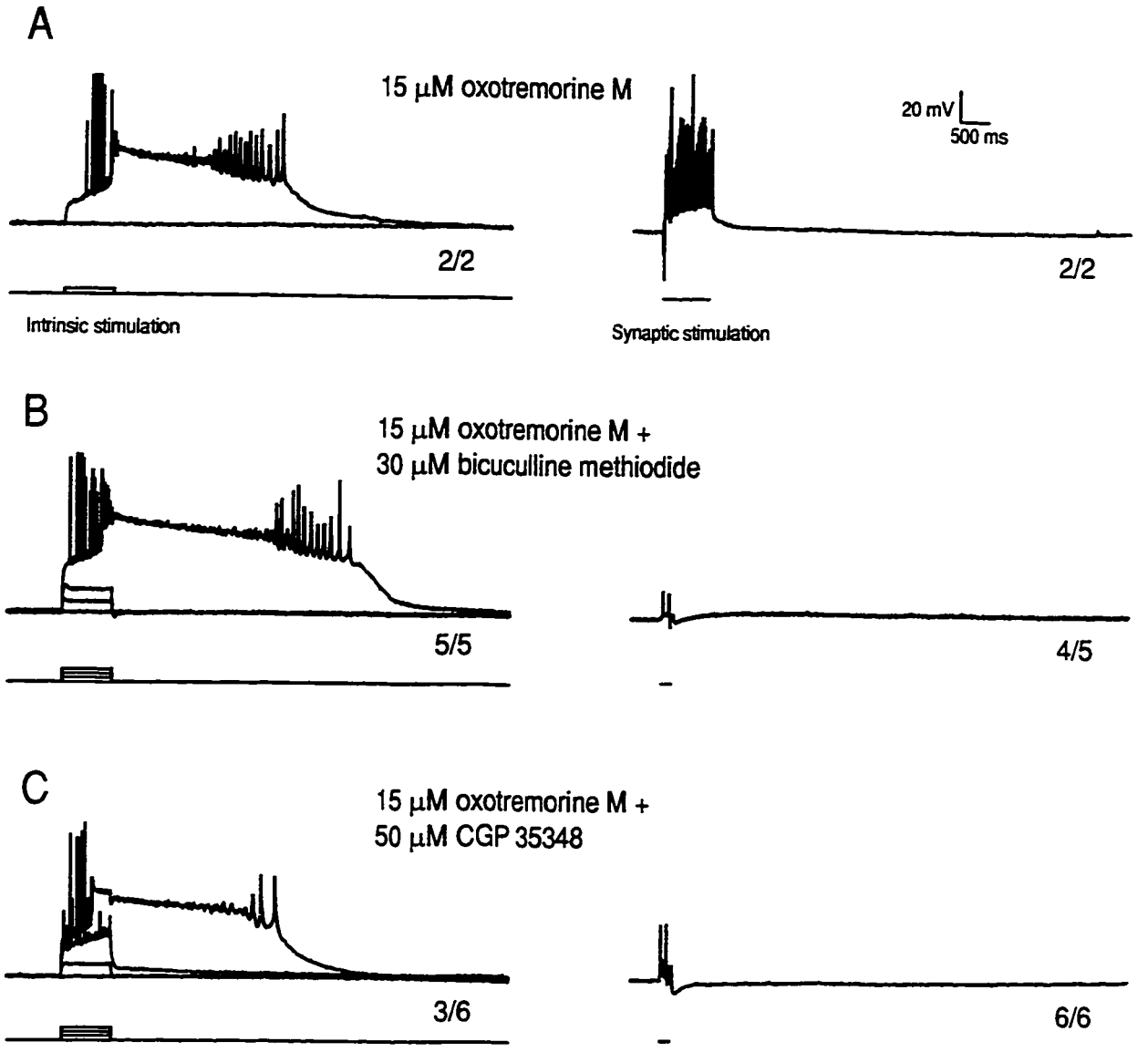

Figure 4

A. Following the application of oxotremorine M, the plateau potential is evoked with intrinsic stimulation in two cells while it is not evoked synaptically in the same two cells.

B. The co-application of bicuculline methiodide and oxotremorine M is not sufficient for the synaptic activation of the plateau potential in 4 out of 5 cells tested.

C. The plateau potential can not be evoked in any of the 6 cells tested following co-application of CGP 35348 and oxotremorine M.

The numbers under each trace indicate the number of cells in which the response displayed was recorded versus the number of cells tested under the described conditions.

Following these attempts to test some of the inhibitory mechanisms that might be acting pre-synaptically to reduce the probability of evoking the plateau potential with synaptic stimulation, I next turned to other sources of inhibition in area CA1 that might be affecting the generation of the plateau potential synaptically.

Does recurrent inhibition prevent synaptic generation of the plateau potential?

An extensive GABAergic interneuron population can be found throughout all of the layers of the hippocampal CA1 region. All three of the major interneuron types found in CA1, the basket cells, O/A interneurons and L-M interneurons, receive extensive excitatory input from the afferents passing along the Schaffer collaterals. Stimulation of the Schaffer collaterals activates both the interneuron and pyramidal cell population in CA1 in a feed forward manner, releasing GABA at inhibitory synapses on pyramidal neurons. Additionally, the presence of afferent connections from CA1 pyramidal neurons onto basket cells and O/A interneurons activates these cells in a feedback manner. The activation of this extensive interneuron population during Schaffer collateral stimulation could potentially prevent the synaptic generation of the plateau potential through GABAergic inhibition. This led to the hypothesis that a reduction in GABAergic inhibition is necessary to reliably reveal the plateau potential following synaptic stimulation. $GABA_A$ receptor mediated inhibition, and its ability to counter fast, excitatory neurotransmission, was the most likely mechanism blocking the synaptic generation of the plateau potential. Experiments in which the $GABA_A$ receptor antagonist bicuculline methiodide and oxotremorine M were co-applied, the plateau potential was synaptically evoked in one out of five cells but was seen in all five of the five cells tested following intrinsic stimulation

(Figure 4B). $GABA_B$ receptor mediated inhibition also plays a large role in regulating neuronal excitability and its potential role in regulating the expression of the synaptically evoked plateau potential warranted experimental testing. Blocking the effects of GABA at $GABA_B$ receptors, with the specific antagonist CGP 35348, while co-applying oxotremorine M failed to uncover the synaptically activated plateau potential in any of the six cells that were tested. The plateau potential could be generated with intrinsic stimulation in three of these six cells (Figure 4C). It did not appear that a failure to reach a membrane potential depolarization threshold prevented synaptic activation from evoking the plateau potential (see Figure 5A)

The single instance where the plateau potential was synaptically activated following bicuculline application and cholinergic receptor stimulation supported the idea that a reduction of inhibition was required to reveal the plateau potential. Although CGP 35348 application, or blocking $GABA_B$ receptors, had no effect on the successful activation of the plateau potential synaptically, it was possible that both $GABA_A$ and $GABA_B$ receptors were acting in concert to prevent the synaptic generation of the plateau potential. The next question to test was; can the plateau potential be synaptically activated following cholinergic stimulation and pharmacological block of both $GABA_A$ and $GABA_B$ receptors? In an attempt to answer this I co-applied oxotremorine M, bicuculline methiodide, and CGP 35348 to the slice prior to stimulation of the Schaffer collaterals. This combination of muscarinic stimulation and $GABA_A$ and $GABA_B$ antagonism was sufficient to reliably reveal the synaptically activated plateau potential in 21 of the 24 cells that a plateau potential was also intrinsically evoked (Figure 5C). In contrast the plateau potential was never evoked in control conditions (see Figure 7A for an example), when the extracellular

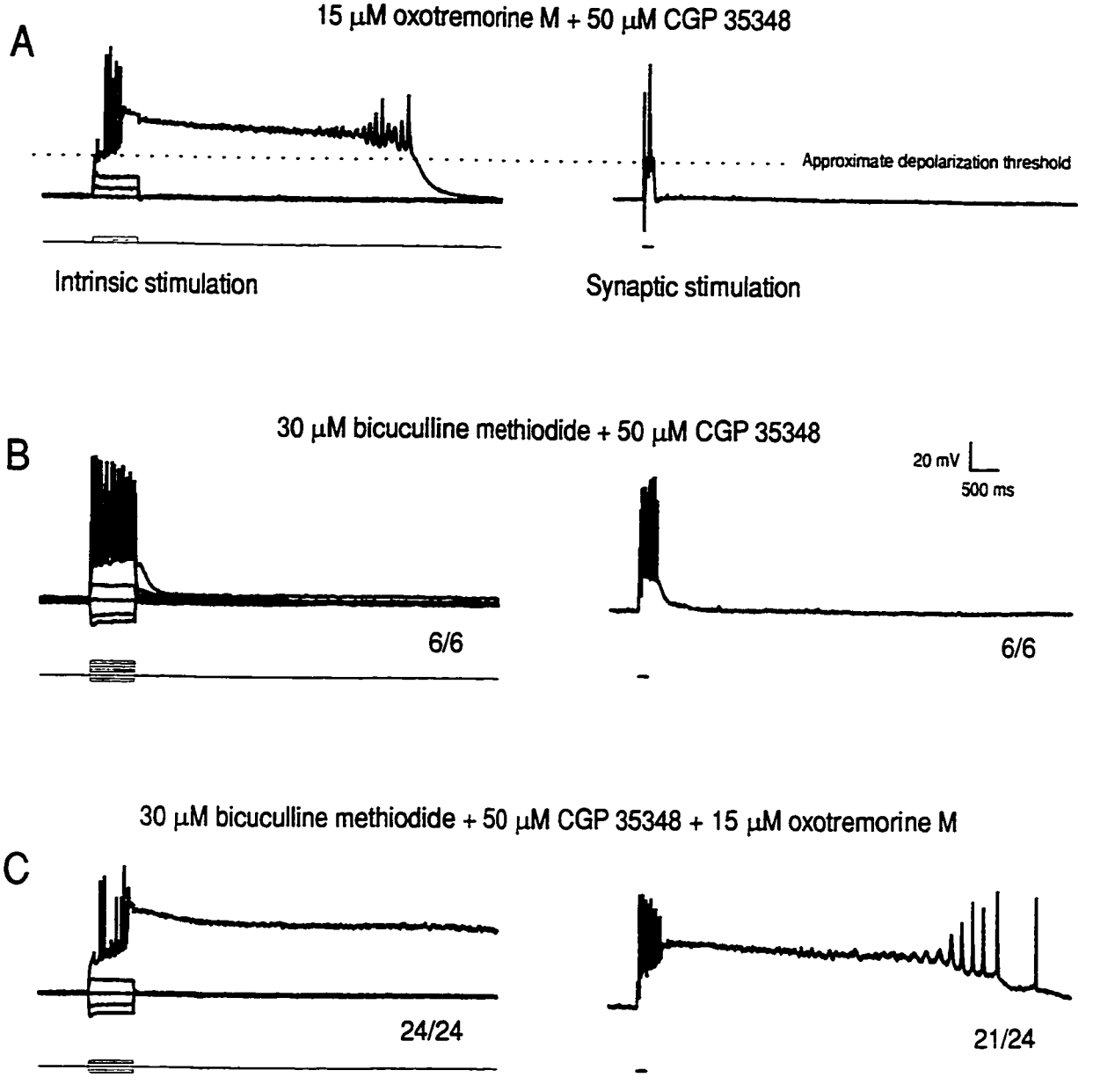

Figure 5

A. A similar level of membrane depolarization is achieved with both intrinsic and synaptic stimulation. Synaptic stimulation fails to evoke the plateau potential in the presence of oxotremorine M and CGP 35348 while intrinsic stimulation is sufficient.

B. In the presence of both bicuculline methiodide and CGP 35348, the plateau potential could not be synaptically evoked in all 6 cells that were tested.

C. In the same cell as panel B, and in 21 of the 24 cells tested, the addition of oxotremorine M to the bicuculline methiodide and CGP 35348 was sufficient to reveal both the intrinsically and the synaptically activated plateau potential.

solution consisted of aCSF only, with synaptic or intrinsic stimulation. Recordings of the response to both intrinsic and synaptic stimulation were carried out in control conditions prior to all drug applications.

Driving cells with intrinsic stimulation, while activating cholinergic receptors, leads to the generation of the plateau potential. Fraser also demonstrated that stimulating metabotropic glutamate receptors, through the application of trans-ACPD, followed by positive DC current step injection to produce spike bursting, activated the plateau potential (Fraser,1997). It was necessary to test the effects of co-applying bicuculline and CGP 35348, in the absence of muscarinic stimulation, to ensure that the block of GABA inhibition alone wasn't responsible for the activation of the plateau potential. The reduction in GABAergic inhibition could lead to an increase in the amount of glutamate released for a given level of synaptic stimulation, allowing for a level of mGluR activation critical for plateau potential generation to occur. The role that blocking GABAergic inhibition has on the successful activation of the plateau potential was tested by applying the $GABA_A$ and $GABA_B$ receptor antagonists bicuculline and CGP 35348 in combination. This was found to be insufficient for the generation of the plateau potential following either intrinsic or synaptic stimulation in all 6 of the cells tested (Figure 5B). Recordings from a single cell in Figure 5 help to illustrate the dependence of the synaptically activated plateau potential on both the block of GABA receptors and muscarinic stimulation. In the presence of both GABA receptor antagonists, a high level of excitation can be seen in the recorded cell following synaptic excitation. A membrane potential depolarization that continues for a few hundred milliseconds beyond the stimulation of the Schaffer collaterals can be seen. Intrinsic or synaptic stimulation are not sufficient for the generation of a long duration

depolarization in the presence of these two GABA receptor antagonists. However, once oxotremorine M is added to the perfusing bath, the plateau potential can be reliably evoked with both intrinsic and synaptic stimulation. As recorded in all five of the cells tested, blocking GABAergic inhibition alone doesn't allow for the plateau potential to be synaptically generated. The addition of a muscarinic agonist to the GABA receptor antagonists reveals the synaptically evoked plateau potential consistently.

Intrinsic versus synaptic generation:

A prolonged depolarization of the membrane potential can be synaptically evoked in CA1 pyramidal cells when muscarinic stimulation is applied to the slice preparation and GABAergic inhibition is blocked at $GABA_A$ and $GABA_B$ receptors. Can it be demonstrated that this depolarization and the plateau potential reported by Fraser and MacVicar are the same thing, when different conditions are required for synaptic versus intrinsic activation? There are no tools that allow us to block the non-specific cation conductance that is believed to underlie the charge movement critical to the plateau potential's generation (Fraser and MacVicar,1996a). The plateau potential can be elicited in the same cell using intrinsic and synaptic stimulation thus allowing for a comparison of the waveform in both cases to be made. In Figure 6A, recordings of the plateau potential from the same cell in which synaptic and intrinsic stimulation were used are overlaid. The similarity of the waveform in both instances helps to demonstrate that both of these cholinergic-dependent events are the same phenomena with different triggering stimuli. If the plateau potential is supported by an increase in conductance through a non-specific cation channel the change in conductance that accompanies the membrane depolarization should be measurable. To

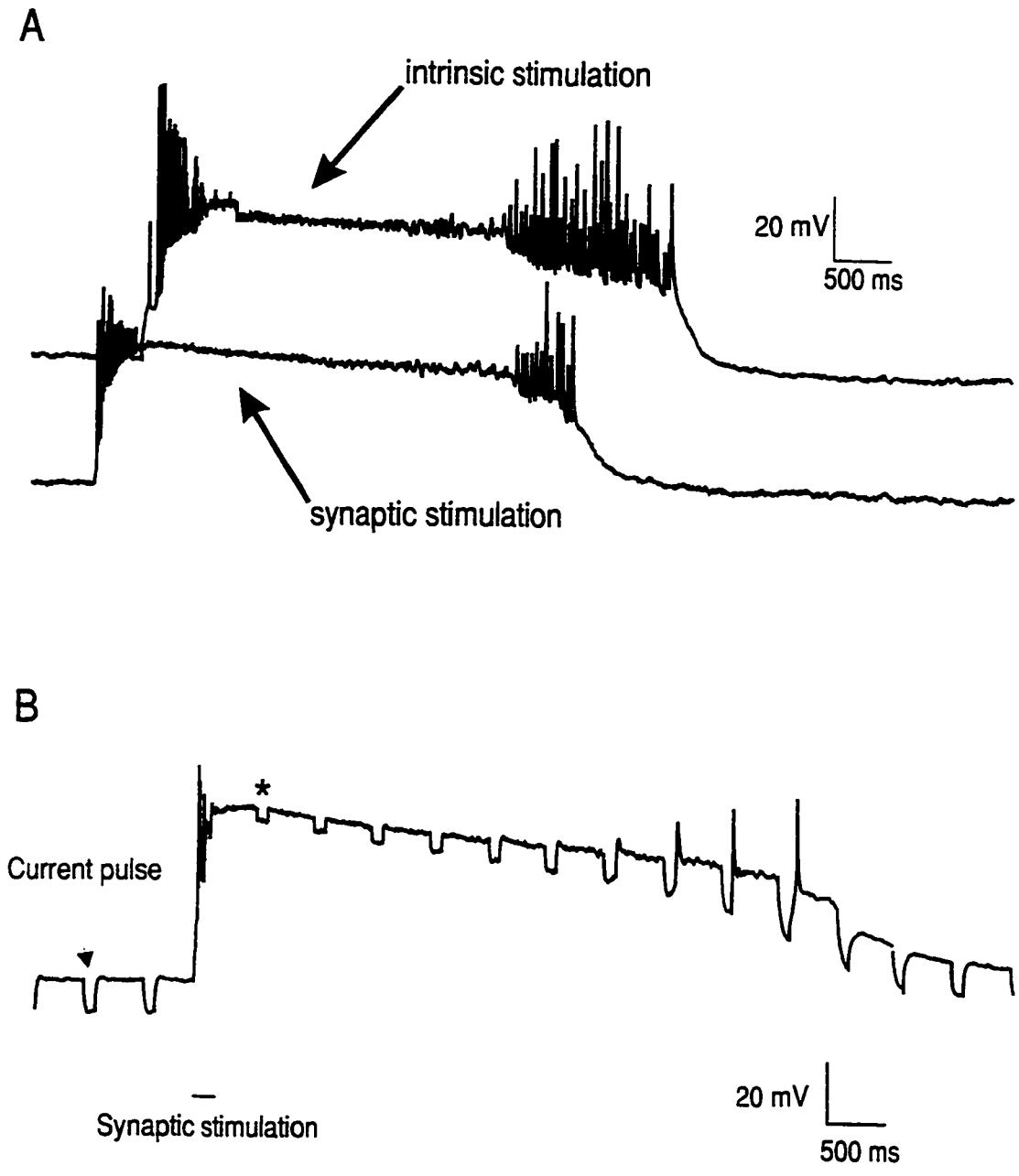

Figure 6

A. Recordings of an intrinsically and synaptically evoked plateau potential from the same cell are juxtaposed.

B. Current pulses (-100 pA, 4Hz, 30ms) are applied before and after synaptic stimulation is applied. Comparing the voltage deflection before (arrow) and immediately after (*) the plateau potential is generated clearly shows a reduction in the input resistance of the cell during the plateau potential.

test this idea, the voltage response of the cell to constant pulses of a set current amplitude were measured. Assuming that the access resistance of the cell was constant during the recording any change in the voltage response to a fixed current pulse would indicate a change in the cell's membrane conductance. In Figure 6B, -100 pA, 4 Hz, 30ms current pulses are injected through the whole cell electrode just prior to and during the application of synaptic stimulation that evokes the plateau potential. In agreement with Fraser and MacVicar's description of the intrinsically evoked plateau potential, I was able to record a decrease in the voltage deflection, or increase in membrane conductance, during the initial depolarization of the synaptically evoked plateau potential (Fraser and MacVicar,1996a)

Is the plateau potential an intrinsic property of neurons in the CA1 region?

Because of the tri-synaptic circuit of the hippocampus, events recorded in the CA1 area may be influenced or even driven by activity in the dentate gyrus and CA3 regions. By electrically stimulating the Schaffer collaterals, calcium dependent release at CA3 - CA1 synapses can be evoked. Such electrical stimulation of the CA3 axonal fibers can also antidromically activate the CA3 cell population. It is necessary to consider how population activity among the CA3 pyramidal cells might affect the level of excitation of CA1 pyramidal cells under the conditions we are using. Applying carbachol (50 μM) to the hippocampal slice can induce short duration oscillatory bursts, or with the addition of $GABA_A$, NMDA and AMPA receptor antagonists, can produce extended periods of rhythmic oscillation within the CA3 pyramidal cell population (Bianchi and Wong,1994; MacVicar and Tse,1989; Williams and Kauer,1997). This carbachol induced rhythmic

bursting appears in both CA3 and CA1 pyramidal cells at the same, synchronized frequency when monitored with paired recordings from neurons in both areas. When a knife cut severs the synaptic connections between the CA1 and CA3 regions, the rhythmic bursting continues in pyramidal cells of the CA3 region but is abolished in the CA1 neurons (Bianchi and Wong,1994; Williams and Kauer,1997). It was important to determine whether the prolonged depolarization generated in CA1with synaptic activation arose independently within area CA1 or was being driven by rhythmic bursting in area CA3. To disconnect the primary afferents passing into the CA1 region from the CA3 region I made a knife cut as indicated (see Figure 7). I placed the bipolar stimulating electrode on the Schaffer collaterals, proximal to the CA1 region beside the knife cut, in order to evoke synaptic release at the CA3-CA1 synapse without antidromically stimulating neurons in area CA3. As shown in Figure 7, in three out of three cells, the knife cut did not prevent the synaptic activation of the plateau potential in the CA1 region. This helps to confirm that this sustained depolarization arises from properties intrinsic to the CA1 cell population .

Under the appropriate conditions, the plateau potential can be reliably evoked with intrinsic or synaptic stimulation. Using intrinsic current injection as a trigger, the plateau appears as an all-or none event with a duration that is dependent on the membrane potential of the cell (Fraser and MacVicar,1996a). Is the synaptically evoked plateau potential an all-or-none event that is triggered by supra-threshold stimulation? To answer this question I attempted to generate the plateau potential while modifying the parameters of Schaffer collateral stimulation in the presence of oxotremorine M, bicuculline methiodide, and CGP 35348. As shown in Figure 8, the plateau potential is generated synaptically in an all or none fashion. Changes in the amplitude, duration, or frequency of synaptic stimulation reveal a threshold

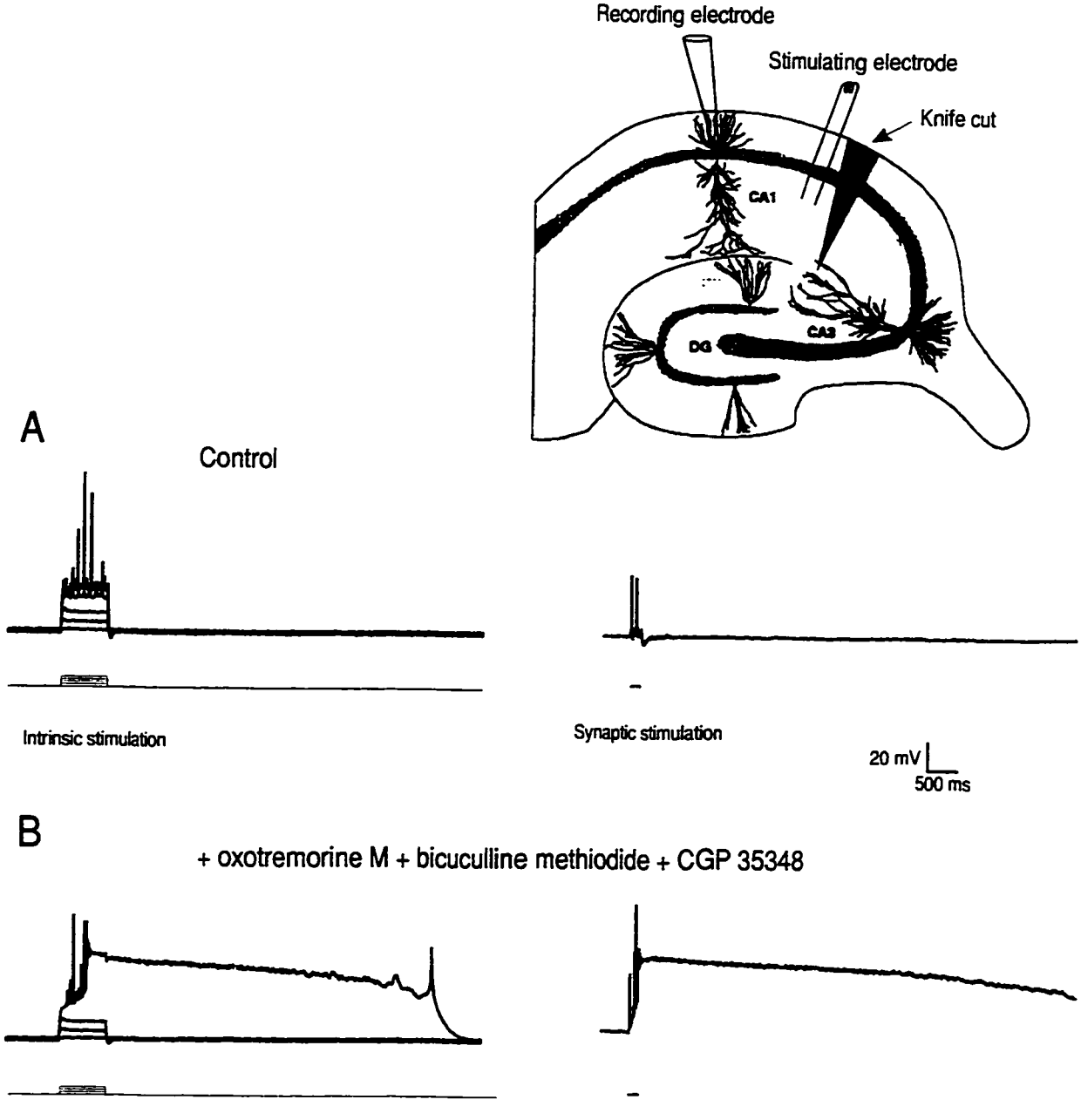

Figure 7

A. In control conditions, intrinsic and synaptic stimulation don't generate the plateau potential.

B. With a knife cut separating the CA3 and CA1 regions, both intrinsic and synaptic stimulation generate the plateau potential in the presence of muscarinic receptor stimulation and $GABA_A$ and $GABA_B$ receptor blockade (as seen in 3 out of 3 cells tested).

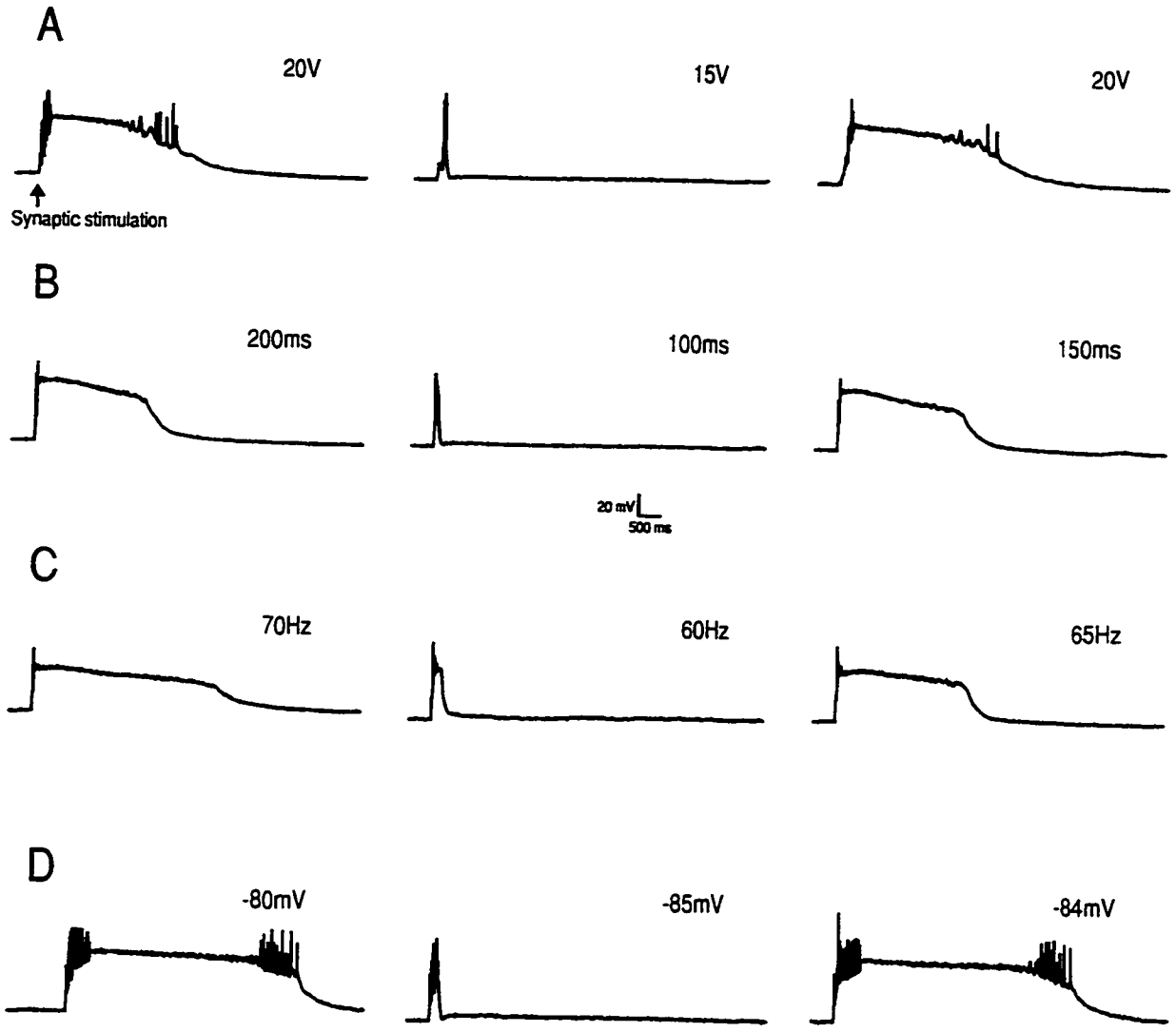

Figure 8

All recordings done in 15 μM oxotremorine M, 30 μM bicuculline, and 50 μM CGP 35348

A. The amplitude of the synaptic stimulation is switched between 20 volts and 15 volts, demonstrating that an activation threshold exists.

B. Varying the duration of the synaptic stimulation reveals a threshold for the generation of the plateau potential.

C. The frequency of the synaptic stimulation can be varied to reveal a plateau potential activation threshold.

D. Hyperpolarizing the membrane potential demonstrates a threshold for the all-or-none plateau potential.

for synaptic stimulation below which a robust depolarization of the membrane potential fails to sustain a long lasting depolarization. The all-or-none nature of this phenomena is also seen when the membrane potential is maintained above or below a threshold level, with a 1 mV change delineating this threshold (Figure 8D).

Calcium entry:

The cholinergic-dependent plateau potential is dependent on an influx mediated increase in intracellular calcium concentration. The plateau potential can not be evoked in a low calcium (0.1mM) extracellular solution, when HVA (high voltage activated) calcium channels are blocked with extracellular cadmium, or when the recorded neuron is perfused with a high concentration of BAPTA intracellularly (Fraser and MacVicar,1996a). Demonstrating that the synaptically evoked plateau potential was also dependent on calcium influx or an increase in the intracellular calcium concentration would further establish that a similar mechanism generates the plateau potential intrinsically and synaptically. As calcium dependent release is crucial for synaptic transmission to take place, I was unable to test the calcium influx dependence of the synaptically activated plateau potential by lowering extracellular calcium or blocking calcium influx through HVA channels. Instead I relied on the intracellular perfusion of a calcium chelator, BAPTA (10 mM), to buffer increases in intracellular calcium and reduce the effects that calcium might have as an intracellular messenger. In 4 out of 4 neurons perfused with a high BAPTA concentration intracellular solution (see Table 1), the plateau potential could not be elicited intrinsically or synaptically following the bath application of oxotremorine M, bicuculline, and CGP 35348 (see Figure 9). Recordings made using a 1.1 mM EGTA

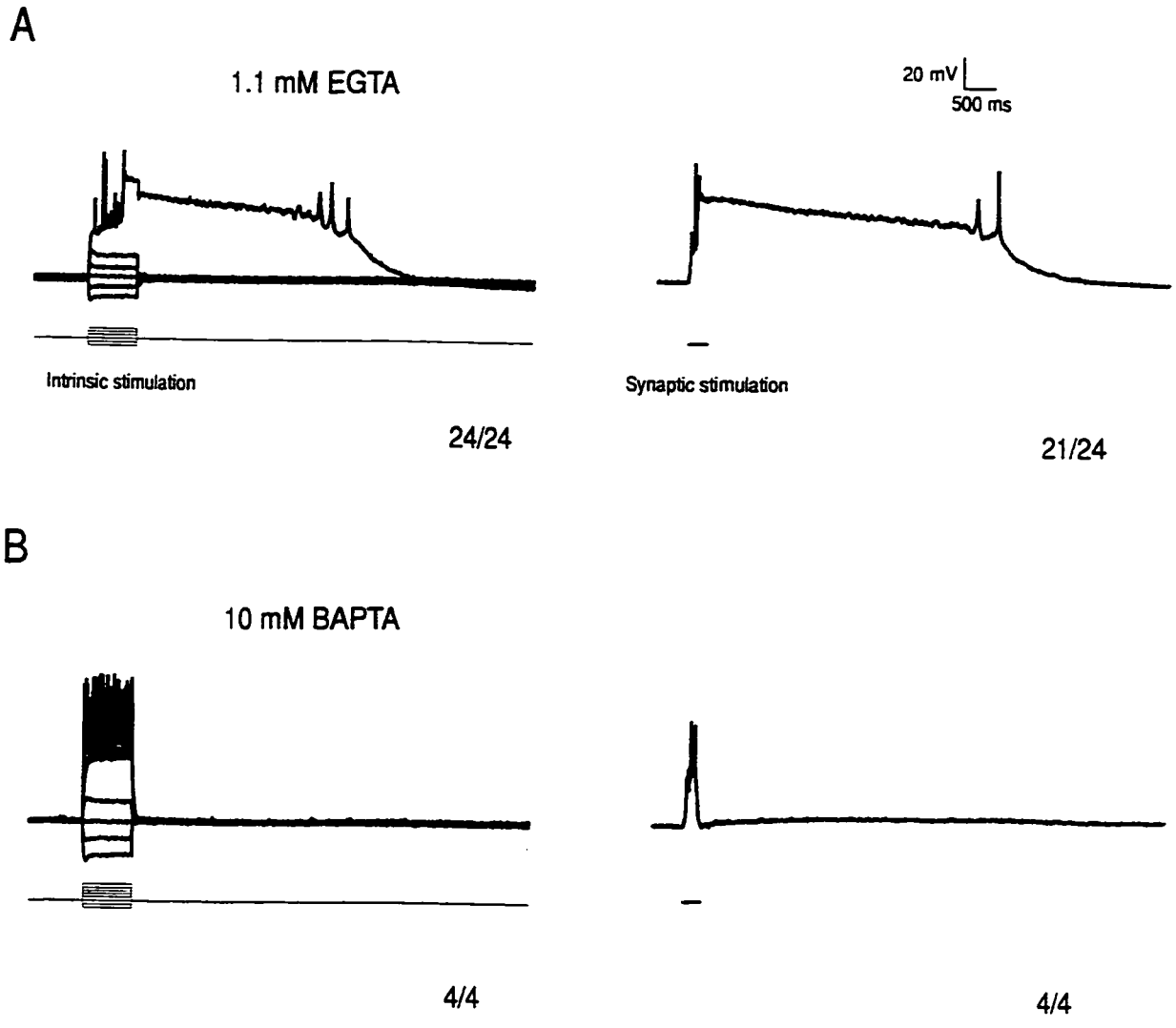

Figure 9

A. Following perfusion with a 1.1 mM EGTA containing intracellular solution, the plateau potential is evoked with both intrinsic and synaptic stimulation (23 out of 25 cells tested).

B. Intracellular perfusion with a 10 mM BAPTA containing solution prevents the generation of the plateau potential following both intrinsic and synaptic stimulation (4 out of 4 cells tested)

The numbers under each trace indicate the number of cells in which the response displayed was recorded versus the number of cells tested under the described conditions.

containing K-gluconate solution in slices from the same animal as the first two BAPTA experiments confirmed that the plateau potential could be generated in these slices in the presence of oxotremorine M, bicuculline, and CGP 35348 (2/2).

Specific points of calcium entry:

It was not possible to apply cadmium extracellularly to globally block HVA calcium channels because of the effect on calcium dependent synaptic release. Fraser and MacVicar have shown that blocking L-type calcium channels with nimodipine or N-type calcium channels with ω-conotoxin GVIA is sufficient to prevent the intrinsic generation of the plateau potential (Fraser and MacVicar,1996a). Because the L-type HVA calcium channel does not play a large role in synaptic release, I was able to test its role in the synaptic generation of the plateau potential (Wu and Saggau, 1994; Wheeler et al., 1994). Application of nimodipine (10 μM) to three cells or nifedipine (1 μM) to one cell produced no change in the ability to synaptically evoke the plateau potential (Figure 10A). This key discrepancy between the synaptically and intrinsically activated plateau potential doesn't necessarily indicate that they are generated by different mechanisms. Along the somato-dendritic axis, L-type channels are found clustered principally around the soma and proximal dendrites while other types of calcium channel, N, R and T type, are distributed more heavily along the distal dendrites. It is possible that intrinsic stimulation activates calcium entry in close proximity to the recording electrode at the cell body and along the proximal dendritic regions. Synaptic contacts along the more distal portions of the dendrites

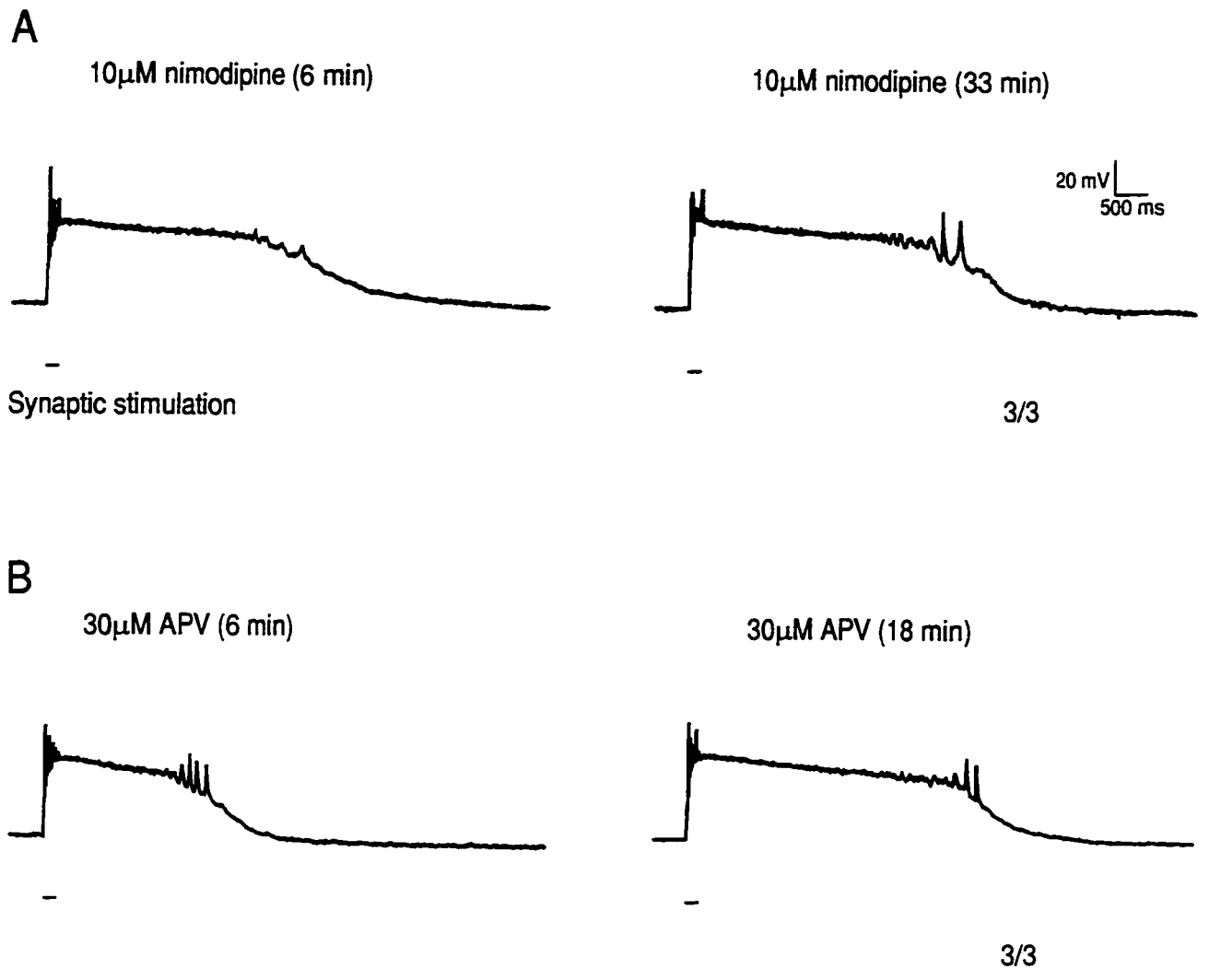

Figure 10

A. Extracellular application of the L-type calcium channel antagonist nimodipine failed to prevent the synaptic activation of the plateau potential (observed in 3 out of 3 cells tested).

B. In a different cell, the addition of the NMDA receptor antagonist APV to the extracellular solution failed to prevent the synaptic generation of the plateau potential (observed in three out of three cells tested).

might cause a different subset of calcium channel to be activated during synaptic stimulation.

The calcium permeability of NMDA receptor gated channels and their location along CA1 pyramidal cell dendrites implicates them as a source of calcium entry during the synaptic generation of the plateau potential (Spruston et al.,1992). The finding that muscarinic receptor activation potentiates NMDA receptor mediated calcium entry fits well with this conductance playing a key role in the calcium influx necessary for the activation of the plateau potential (Hashiguchi et al., 1997). This led to the hypothesis that NMDA receptor channel activation, and the subsequent influx of calcium through these channels, was critical to the synaptic generation of the plateau potential. This was tested by applying the NMDA receptor antagonist APV to the slice preparation, in the presence of bicuculline, CGP 35348, and oxotremorine M, at a concentration that I had previously established abolished synaptically evoked NMDA mediated EPSCs. In the three cells that I tested, there was no change in the ability to synaptically evoke the plateau potential in the presence of 30 μM APV, even when the drug was washed in for over 15 minutes (Figure 10B). The results from experiments in which nimodipine, nifedipine, or APV were employed to block calcium entry lead to the conclusion that calcium influx through either L-type calcium channels or through the NMDA receptor gated channel is not necessary for the synaptic activation of the cholinergic-dependent plateau potential.

Chapter 3

Discussion

I have demonstrated that the cholinergic-dependent plateau potential can be evoked synaptically in CA1 neurons of the hippocampal slice. I began with the belief that it would be a simple matter of stimulating a robust synaptic response in a CA1 pyramidal neuron in the presence of carbachol to generate the plateau potential synaptically. It was evident early on that there was more to the situation when synaptic rather than intrinsic stimulation was used. GABAergic inhibition plays a key role in preventing the plateau potential from being synaptically activated in this model. Additionally, a threshold level of stimulation parameters must be met before a plateau potential can be synaptically evoked.

Synaptically activating GABAergic inhibition:

When synaptic stimulation was used to activate the plateau potential, my results indicated that GABAergic inhibition had a robust effect, preventing the generation of the plateau potential. This is not surprising considering the method of synaptic stimulation. I was not discretely stimulating a few fibers that were selectively activating the neuron I was recording from. Rather, I was stimulating a large cross section of the fiber bundle passing from area CA3 to CA1 causing the excitation of neurons throughout the pyramidal cell and interneuron population (Collingridge et al.,1983;). GABAergic interneurons throughout the CA1 area were being synaptically activated, resulting in both feed-forward and feedback inhibition (Bragin et al.,1997; Buzsaki,1984).

Inhibition and epilepsy, a link to the plateau potential:

My results indicate that a reduction in GABAergic inhibition is necessary for the reliable synaptic activation of the plateau potential. In "epileptic" slice preparations, whole animal models, and human patients, a reduction in GABAergic inhibition is often associated with the development of seizure activity (Bradford,1995; Thompson,1994). The finding that GABAergic inhibition is strongly involved in controlling the synaptic activation of the plateau potential strengthens the possibility that the plateau potential plays a key role in the pathophysiology of epilepsy. GABAergic inhibition has been shown to play a role in controlling the onset of seizure activity in many epileptic models and human patients (Bradford,1995; During et al.,1997; Gibbs et al.,1997; Mangan and Lothman,1996) . The transition to seizure bursting, when monitored with surface or depth recordings, is seen as a sudden change from the shorter duration interictal discharge pattern to more sustained ictal depolarizations. This rapid transition shares some similarities with the sudden, all-or-none nature of plateau potential onset; both in the sharp onset of the prolonged depolarization and the duration of the tonic ictal depolarization and plateau potential. The synaptically activated plateau potential has an activation threshold that is based on the level of synaptic input received from Schaffer collateral afferents. It is possible that a discrete level of excitatory output from area CA3 is required to trigger the rapid appearance of the plateau potential, or ictal depolarization, in CA1 pyramidal neurons during seizure activity *in vivo*.

In addition to a threshold of synaptic input, the synaptically activated plateau potential is dependent on muscarinic receptor stimulation. In my slice model, where Schaffer collateral

stimulation activated the glutamatergic afferents passing from area CA3 to CA1, muscarinic activation depended on exogenous application of the receptor agonist oxotremorine M (Collingridge et al.,1983). It has recently been shown that stimulating the fibers that pass through the stratum oriens, induces a slow EPSP in CA1 pyramidal cells, even after $GABA_{A+B}$, AMPA, and NMDA receptors are blocked (Morton and Davies,1997). This depolarization is sensitive to atropine and is enhanced by the acetylcholinesterase inhibitor physostigmine. The waveform of this synaptically activated depolarization and the synaptically generated plateau potential are similar in many respects, including the level and duration of the depolarization. The fibers that pass through the stratum oriens in the hippocampal slice originate in the septum *in vivo*, releasing acetylcholine in the hippocampus at their terminal ends (Nyakas et al.,1987; Frotscher and Leranth,1985). Additionally inhibitory interneurons in the hippocampal CA1 region receive inhibitory inputs from GABAergic afferents that originate in the septum. The resulting reduction in hippocampal interneuron activity causes functional disinhibition of hippocampal pyramidal neurons (Freund and Antal,1988; Toth et al.,1997). Both the synaptically activated plateau potential and the slow EPSP of Morton and Davies are dependent on the pharmacological block of GABAergic receptors. It is conceivable that *in vivo*, a high level of activity in the medial septum could bring about simultaneous activation of muscarinic receptors on CA1 pyramidal neurons and inhibition of GABA release from hippocampal interneurons. This could provide an environment more favorable to the activation of the plateau potential in the hippocampus. Future experiments could utilize the septal-hippocampal slice preparation to test whether stimulation applied in the MSDB, and the subsequent activation of the cholinergic and GABAergic fibers passing from there to the hippocampus, is capable of evoking the plateau potential in CA1 pyramidal neurons.

The calcium dependence of the plateau potential:

Whether it is intrinsically or synaptically activated, the plateau potential is dependent on an increase in intracellular calcium (Doll et al,1996; Fraser and MacVicar,1996a). Intracellular perfusion of 10 mM BAPTA blocked the synaptically generated plateau potential in four of the four cells tested while the plateau potential could be synaptically evoked in 21 of 24 cells tested following intracellular perfusion of 1.1 mM EGTA. One possible explanation for the difference in effect between BAPTA and EGTA addition is the nine-fold higher concentration of BAPTA added to the intracellular solution compared with EGTA (see Table 1). However, in experiments designed to test the neuroprotective effects of buffering calcium to prevent glutamate dependent neurotoxicity, Tymianski et al. found that BAPTA was far more effective than EGTA, even when both were applied in equal concentrations (Tymianski et al.,1994). Both EGTA and BAPTA have similar affinities for calcium (K_d of approximately 100 nM) but the forward calcium association rate is approximately 400 times greater for BAPTA than EGTA (Tymianski et al.,1994). The faster association rate of BAPTA allows it to buffer calcium ions much more effectively than EGTA in microdomains containing high calcium (Tymianski et al.,1994) This property may be important in quenching the effect of calcium influx at the hot spots surrounding voltage gated calcium channels and calcium permeable non-specific cation channels during the generation of the plateau potential.

The plateau potential is driven by mechanisms intrinsic to the CA1 region:

The results from these BAPTA experiments also help to confirm that the synaptically activated plateau potential arises post-synaptically in the recorded cell. Additional evidence that the plateau potential arises from properties intrinsic to the CA1 pyramidal cell population comes from the knife cut experiments. When the CA3 cell population was synaptically isolated from the neuronal population in CA1, the plateau potential could still be activated in area CA1. This finding helps us to define the plateau potential as a phenomenon that arises in the CA1 region due to properties intrinsic to the resident neuronal population. Much of the work on seizure discharge in the hippocampus has focused on the CA3 region as the bursting generator with area CA1 simply acting as a mirror for the activity in area CA3 (Bianchi and Wong 1994; Dichter and Spencer 1969; Rafiq et al.,1993). It seems more likely that discharge within the CA3 region acts as a trigger for episodes of prolonged depolarization in area CA1, where the plateau potential arises independently in pyramidal cells.

Localizing the synaptically activated plateau potential:

I have shown that application of L-type calcium channel antagonists fails to block the synaptically activated plateau potential. In addition to eliminating a criterion that helps to define the plateau potential when intrinsic stimulation is used, this information helps us to delineate the site of plateau potential generation along the somato-dendritic axis. It has been shown that L-type calcium channels are distributed principally around the soma and proximal dendrites of CA1 pyramidal cells (Miura et al.,1997; Westenbroek et al., 1990).

Blocking calcium entry through this channel subtype effectively abolishes the intrinsically, but not the synaptically, evoked plateau potential. Injecting DC current at the soma may generate calcium spiking in the soma which then propagates into the dendrites (Miura et al.,1997). Blocking the primary source of calcium entry on the cell body and proximal dendritic areas with nimodipine may prevent the generation of calcium spikes at the soma following DC current injection. During synaptic activation of CA1 pyramidal cells, calcium influx occurs primarily through voltage gated calcium channels along the length of the dendrites, with the lowest calcium signal seen at the soma (Miykawa et al.,1992). I have demonstrated that the calcium influx that leads to synaptic generation of the plateau potential is not dependent on NMDA receptor or L-type calcium channel activity. It is interesting to note that Morton and Davies, who describe a prolonged depolarization ($EPSP_m$) in CA1 pyramidal cells driven by acetylcholine release, also found that blocking L-type calcium channels, with nitrendipine, had no effect on their ability to evoke the $EPSP_m$ (Morton and Davies,1997). They concluded that the plateau potential described by Fraser and MacVicar made no contribution to their $EPSP_m$, where the use of synaptic stimulation rather than intrinsic stimulation and the subsequent activation of different calcium conductances may have lead to the difference in findings (Morton and Davies,1997). The calcium entry underlying the synaptic activation of the plateau potential is probably occurring in more distal regions of the dendrites (greater than 100 μm from the soma), where high voltage activated (HVA) R-type and low voltage activated (LVA) T-type channels are found in abundance (Magee and Johnston,1995b). The recent isolation of SNX-482, a selective R-type calcium channel antagonist will allow for the role of R-type channels in the synaptic generation of the plateau potential to be tested in future

experiments (Urge et al.,1997). Fraser and MacVicar also demonstrated that blocking N-type calcium channels with an application of ω-conotoxin GVIA prevented the intrinsic activation of the plateau potential (Fraser and MacVicar,1996a). This is to be expected when the fairly uniform distribution and robust level of expression of N-type calcium channels at the soma and over the length of the dendritic processes in CA1 pyramidal cells is considered (Mills et al.,1994). Because N and Q-type calcium channels play a large role in presynaptic calcium entry and subsequent transmitter release from CA3 axon terminals I was not able to test their involvement in the synaptic activation of the plateau potential (Wu and Saggau,1994; Wheeler et al.,1994).

Calcium and the CAN channel:

The primary charge carrier underlying the plateau potential, following calcium dependent activation, is believed to be the non-selective cation conductance, or CAN channel (Crepel et al,1994; Fraser and MacVicar,1996a). Although it has not been isolated with molecular techniques and localized, it is conceivable that the CAN channel and voltage gated calcium channels co-localize along the distal arbors of apical dendrites in CA1 pyramidal cells. The plateau potential is a regenerative event, where continual activation of HVA calcium channels and depolarizing ion movement through the CAN channel are both required to sustain the prolonged depolarization (Fraser and MacVicar,1996a). This close functional cooperation between the CAN channel and dendritic calcium conductances during the generation of the plateau potential may necessitate close physical proximity between these two types of channel. It is possible that the CAN channel is distributed along the distal

apical dendrites of pyramidal CA1 neurons, where it is activated by calcium influx through adjacent voltage gated channels during synaptic stimulation. Calcium entry in these more distal regions is activated by both sodium spike-linked and non-spike events, where voltage gated calcium channels open in response to both sodium-dependent action potentials and sub-threshold (for action potential firing) membrane depolarizations (Miyakawa et al.,1992). The highest level of synaptically evoked, spike-linked calcium entry occurs close to the soma, only slightly more distal to the region where somatic current injection causes calcium entry (Miyakawa et al.,1992). Synaptic stimulation is also able to generate non-spike dependent calcium entry, probably due to direct activation of voltage dependent calcium channels by EPSPs, at more distal dendritic locations (Magee and Johnston,1995a; Miyakawa et al.,1992). This may help to explain why synaptic activation of the plateau potential is not prevented when L-type calcium channels are blocked. There may be non-spike mediated calcium entry from EPSP depolarization at dendritic sites in close proximity to the CAN channels, raising local calcium levels to a threshold sufficient for calcium dependent CAN channel activation. Spike-linked calcium entry may be augmented in these more distal dendritic regions following application of oxotremorine M . A muscarinic dependent increase in calcium entry has been shown at dendritic sites 250 μm from the soma (Tsubokawa and Ross,1997). This increase in the calcium signal in more distal dendritic regions parallels a reversal in dendritic spike amplitude decrease that is also dependent on muscarinic stimulation (Tsubokawa and Ross,1997). The dendritic calcium signal is also modulated by GABAergic inhibition at sites distal to the soma (Tsubokawa and Ross,1996). Blocking $GABA_A$ receptor mediated inhibition augments the calcium signal generated by backpropagating action potentials 300μm from the soma (Tsubokawa

and Ross,1996). In my experiments the application of a muscarinic agonist and a $GABA_A$ receptor antagonist may have allowed for back propagating spikes and spike-linked calcium entry to penetrate further up the dendritic tree, resulting in the activation of a greater population of CAN channels and their associated voltage gated calcium channels.

The ability to localize the sites of calcium influx during the generation of the plateau potential would shed further light on the dendritic location of plateau potential genesis. Future experiments designed to capture video images of a calcium indicator filled CA1 pyramidal neuron in whole cell patch configuration, if acquired at a fast enough rate, should reveal, augmented by oxotremorine M and bicuculline application, a large synaptically driven dendritic calcium signal. With $GABA_{A+B}$ receptors blocked and muscarinic receptors stimulated, a large synaptically evoked dendritic calcium signal should be closely followed by the onset of the plateau potential.

A non-pathophysiological role for the CAN channel?

The non-selective cation conductance, dependent on calcium and a $G_{q/11}$ linked receptor for its activation, may play an important role in the regenerative depolarization underlying the plateau potential (Congar et al,1997; Fraser and MacVicar,1996a). If it is distributed along the distal regions of CA1 pyramidal cell dendrites, in close proximity to voltage gated calcium channels, the CAN channel may play a functional role in dendritic signal propagation. Both dendritic EPSPs and sodium spikes back-propagating from the soma activate voltage gated calcium channels in distal dendritic regions (Magee and

Johnston,1995a; Miyakawa et al,1992; Spruston et al.,1995) CAN channels in the dendrites may assist in boosting the local dendritic propagation of calcium and sodium spike signalling. Calcium influx through a voltage gated channel could cause a rapid increase in calcium concentration in the microdomain surrounding the channel, leading to the facilitation of a closely located CAN channel. At the pre-synaptic terminal of the squid giant axon, the intracellular calcium concentration in the microdomain surrounding an active zone calcium channel cluster (~0.3 μm^2) reaches 200-300 μM for an average 200ms in duration following axonal stimulation (Llinas et al.,1992). Back-propagating spikes passing through a region with facilitated CAN channels could establish local regenerative depolarizing events where calcium and CAN channels would mutually co-activate at a level subthreshold for plateau potential generation. An over expression of this signal amplification, the plateau potential, may arise as a pathological phenomena in the hippocampus, if preceded by both a loss of local inhibitory control and an inappropriate activation of muscarinic and/or metabotropic receptors.

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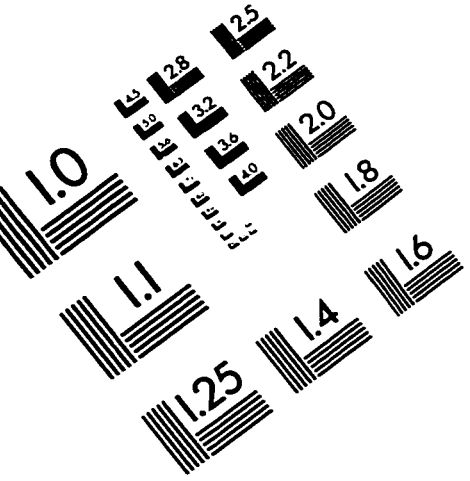
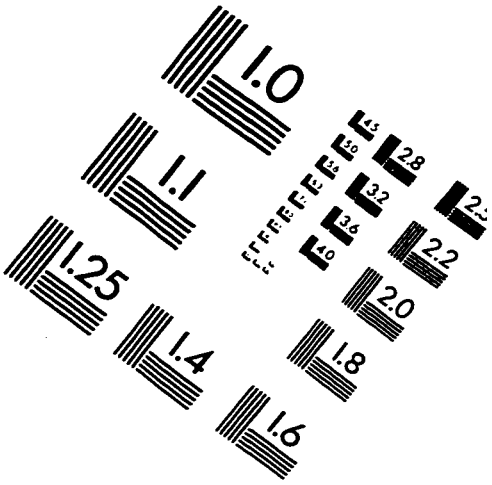
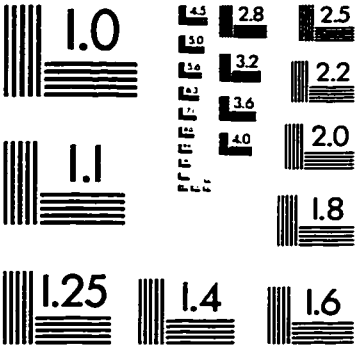
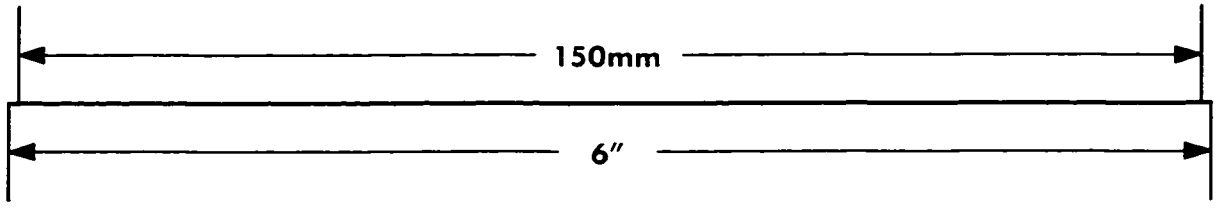
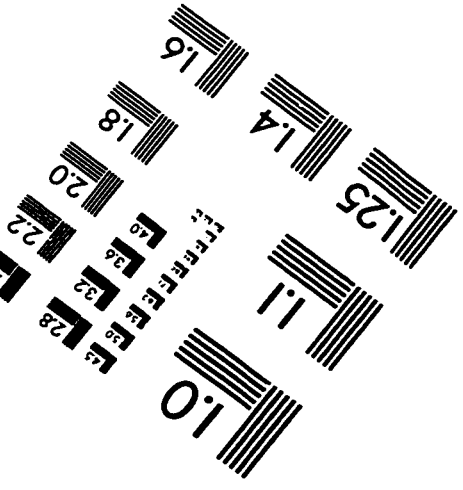
IMAGE EVALUATION TEST TARGET (QA-3)

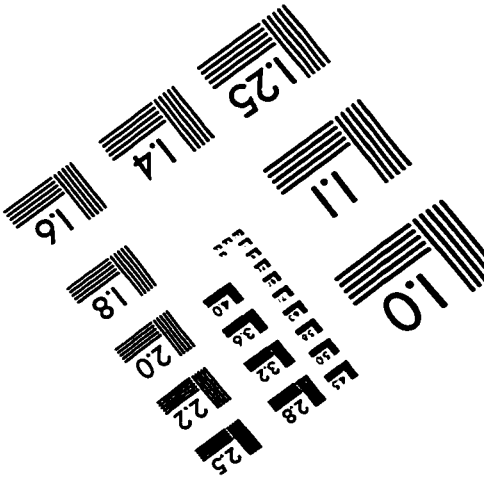












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