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Modulation of [gamma]-aminobutyric acid (GABA) type A receptor-mediated responses in spinal dorsal horn neurons by [mu]-opioid receptor agonists and Ca\(^{2+}\)/calmodulin-dependent protein kinase and Monte Carlo simulation of the GABAergic synaptic transmission

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Modulation of γ-aminobutyric acid (GABA) type A receptor-mediated responses in spinal dorsal horn neurons by μ-opioid receptor agonists and Ca²⁺/calmodulin-dependent protein kinase and Monte Carlo simulation of the GABAergic synaptic transmission

by

Rong Wang

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1995

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CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation consists of four chapters including a general introduction which contains the research objectives and literature review, and followed by a general discussion and a bibliography. The second and third chapters include two published papers (R.A. Wang and M. Randic, 1994, *Neuroscience Letter*; R.A. Wang, M. Kolaj, G. Cheng and M. Randic, 1995, *Journal of Neurophysiology*), concerning the modulation of \( \gamma \)-aminobutyric acid (GABA) type A receptor-mediated responses in rat spinal dorsal horn (DH) neurons by \( \mu \)-opioid agonists and calcium/calmodulin-dependent protein kinase. The fourth chapter involves a study of Monte Carlo simulation of GABAergic synaptic neurotransmission. It first gives the theoretical background in simulation of diffusion and receptor binding. It then presents a simulation scheme followed by its computational validation. Finally, results and discussions of the simulation studies are given that answer some of the questions of interest to biophysicists and electrophysiologists.

The experimental part of the dissertation contains a large part of results obtained by the author during his graduate study for a co-major in neuroscience with Dr. Mirjana Randic. The simulation work was done by the author during his graduate study under Dr. James L. Cornette for a co-major in applied mathematics.
Research Objectives

Modulation of GABA$_A$ receptor-mediated responses of spinal DH neurons

Numerous anatomical and physiological studies have revealed that $\gamma$-aminobutyric acid is one of the major neurotransmitters mediating the inhibitory actions in the spinal DH. The transmitter, by binding to GABA$_A$ receptors, induces a fast inhibitory potential in the postsynaptic neuron. Endogenous opioids have also been implicated in the pre- and postsynaptic inhibitory effects on spinal DH neurons. Moreover, recent evidence indicates that some endogenous opioids coexist with GABA in a large proportion of opioidergic interneurons, especially in the superficial DH. This raises the possibility of interactions between the two inhibitory pathways. The first part of the research was directed towards the possible modulation by opioids of the sensitivity of postsynaptic GABA$_A$ receptors on the acutely isolated spinal DH neurons from superficial laminae.

Molecular and functional studies demonstrated that GABA$_A$ receptor can be regulated through phosphorylation by various protein kinases, including PKA, PKC and tyrosine kinase. A recent study (McDonald and Moss, 1994) reported that purified GABA$_A$ receptor subunits can be phosphorylated by calcium/calmodulin dependent protein kinase (CaM-KII). This suggests a possible functional role of the kinase in GABA$_A$ receptor-mediated response. The second part of the research was designed to address this possibility by testing the effect of intracellular application of CaM-KII on GABA$_A$ receptor-mediated responses in spinal DH.
Monte Carlo simulation of GABAergic synaptic transmission

The inhibitory synaptic transmission at GABA_A synapses is one of the the neuroscience subjects being intensively studied due to the clinical importance of the pathway. Benzodiazepines and barbiturates are found to modulate the function of GABA_A receptors by binding to the regulatory sites on the receptor-ion channel complex. However, there are still a number of unanswered questions regarding the GABA_A inhibitory transmission in vivo. The role of such factors as the concentration of GABA in the synaptic junction, the number of available receptors and the role of receptor desensitization are among the most controversial subjects at GABA_A synapse. It has been recently proposed that the concentration of GABA during the generation of miniature inhibitory postsynaptic currents (mIPSC) might exceed 0.5-1 mM and this high concentration might last for the period of on-rate of mIPSC (Maconochie et al., 1994). It has further been suggested that at peak mIPSC there are about 60-80 GABA_A receptors in the open state and about 80% of receptors are saturated.

Monte Carlo analysis has proven to be a realistic, and illustrative method to simulate synaptic neurotransmission. It is an alternative to solving simultaneous differential equations for neurotransmitter diffusion and receptor binding/unbinding. This study used the idea of Monte Carlo analysis to simulate GABA_A receptor-mediated IPSCs in a model synapse. The focus of the simulations is to answer some of the questions (including the ones discussed above) of interest in the electrophysiological and biophysical studies of the GABAergic neurotransmission. In the mean time, comparisons were made between the approaches using either deterministic differential equations or the Monte Carlo method in the simulation of transmitter diffusion and receptor binding. Error analyses were done and presented as theorems.
Background and Literature Review

Sensory receptors and primary sensory neurons

Sensory information, transduced by peripheral receptors (cutaneous, muscle, visceral), is transmitted to the spinal dorsal horn (DH) through the synapse between primary sensory neurons, located in the dorsal root ganglia (DRG), and spinal cord (Campbell et al. 1989; Martin, 1991; Martin and Jessell, 1991). The synaptic contacts of primary afferent fibers with heterogenous populations of spinal cord neurons represent, as generally accepted, the first synapse in the central nervous system (CNS), where sensory information is integrated and processed.

Primary sensory neurons in the DRG have been classified into two major types with respect to their afferent size, conduction velocity, characteristics of somatic action potentials, sensory modality and neurochemistry. Type A neurons are large cells (30-70 μm in diameter) that stain lightly with basic dyes and silver salts. Type B neurons, on the other hand, are small cells (25-30μm in diameter), having darkly stained perikarya (Liebermann, 1976; Harper and Lawson, 1985a). Large A neurons have short-duration action potentials (0.49-1.35 ms at the base) that are tetrodotoxin-sensitive, and give off large diameter myelinated axons that conduct in Aα- (30-50m/s) or Aδ (14-30m/s)-fiber range. Small type B neurons are characterized by long-duration action potentials (0.5-8.0 ms at the base) that are in some cases are tetrodotoxin-insensitive. In general they give off small diameter axons that are poorly myelinated or unmyelinated and conduct in C(1.4 m/s)- or Aδ(2.2-8.0 m/s)-fiber range. That some of these membrane properties might be related to functional categories was supported by finding that chemoreceptors (conduction velocity=11m/s)
and slow baroreceptors (conduction velocity =10m/s) have long action potentials with a hump, whereas fast baroreceptors (conduction velocity, 33 m/s) have short action potentials with no hump (Belmonte and Gallego, 1983). In important recent studies, different types of mechanoreceptors were identified and correlated with action potential duration, rate of rise, amplitude, afterhyperpolarization, and inward rectification. Interestingly, the physiological parameters correlated better with the type of information transmitted than with the conduction velocity of the axon (Rose et al., 1986; Koerber et al., 1988). However, it has also been reported that C fibers may be given off by either large or small cell body (Hoheisel and Mense, 1986). This means that a specific fiber type may not be simply related to the cell size. Furthermore, for rat DRG cells, Aα, Aδ and some Aβ cells had fast action potentials and some Aβ and C cells had longer action potentials, primarily owing to a hump on the falling limb (Harper and Lawson, 1985b). These last findings are interesting in that the C cells, which are presumably small, and some Aβ cells, which are presumably large, have one kind of action potential and the Aα cells, which are presumably large, and some Aδ cells, which are presumably small and intermediate in size, have another.

**Primary afferents**

Primary sensory neurons are unipolar neurons (sometimes called pseudounipolar since they originated as bipolar cells) (Ranson, 1912). The axons of these neurons divide into two branches that project in opposite directions. The central branch goes to the spinal cord through the dorsal root and the other branch innervates sensory receptors and contributes to the peripheral nerve. Together they form a
primary afferent fiber. The primary afferent fibers are classified based on their sensory modality, diameter, conduction velocity and presence of myelin. The cutaneous afferent fibers (the primary fibers from skin) are alphabetically coded as: Aα, Aβ, Aδ and C (Martin, 1991). The Aα and Aβ fibers are associated with A sensory neurons. They constitute a group of large, myelinated, fast conducting afferents that enter the spinal cord through the medial division of the dorsal roots and project predominantly to laminae III and V (Brown, 1981; Woolf, 1987). The terminals of these afferents generally contain round vesicles and make asymmetric synapses mainly with the dendrites and spines of the second order dorsal horn neurons (Réthelyi, 1983). Aδ- and C-fibers are associated with small type B neurons. They have small diameters and are either myelinated (Aδ) or unmyelinated (C). These fibers enter the spinal cord through the lateral division of the dorsal roots, including the Lissauer's tract, and project mainly to the laminae I, II, and V and to the central canal (Christensen and Perl, 1970; Light and Perl, 1979; McMahon and Wall, 1985; Chung, 1987). Aδ and C fibers terminate in synaptic glomeruli. They establish asymmetric axodendritic synapses and are postsynaptic to axonal and dendritic endings that contain flattened or pleomorphic vesicles (Maxwell and Réthelyi, 1987; Willis, 1991).

Primary afferents that convey distinct somatosensory modalities have specific routes and end in different regions of the spinal cord. In other words, the specific sensory information transduced by a particular mechanoreceptor, thermoreceptor, or nocireceptor in the skin is maintained and processed in different region of the central nervous system. In addition, it is a general rule that fast-conducting myelinated Aα and Aβ fibers have a low threshold of activation. They innervate a variety of low-threshold mechanoreceptors. The slow-conducting unmyelinated fibers C and Aδ
fibers, on the other hand, have a high threshold of activation.

There are many peptides colocalized in DRG cells, and there are some preliminary indications that patterns of localization may be correlated with area of peripheral innervation (Willis, 1991).

**Cytoarchitecture of the spinal dorsal horn**

The cytoarchitecture organizational schemes were presented by Rexed (1952, 1954). The currently accepted scheme was based on the shapes, sizes, densities and distributions of the neuronal cell bodies in the spinal cord. According to the scheme, the spinal cord has been separated into ten different laminae, with laminae I-VI being in the dorsal horn, laminae VII-IX in the ventral horn and lamina X around the central canal (Fig.1.1). The significance of the organizational scheme is supported by the finding that the distribution of primary afferents fits the pattern of laminae. It has also been correlated with physiological studies and extrapolated to almost every mammalian species studied (Paxinos and Watson, 1982).

*Lamina I*

Lamina I is the classical marginal zone. It is the most superficial layer of the dorsal horn. The neuronal cell bodies in lamina I have a wide range of sizes. Two major types are marginal cells and "smaller cells". The best studied marginal cells, which make up a minority of the neurons in the lamina I, are large neurons (10-15×30-50 μm) with flattened cell bodies and horizontal dendrites (Rexed, 1952). Axons of cells in lamina I project mainly to the thalamus or other parts of the spinal cord (Burton and Loewy, 1976). Other important destinations include midbrain and brain stem reticular formation (Brown, 1980; Willis, 1991). There is good evidence
Figure 1.1: Schematic diagram of Rexed’s laminae of spinal cord gray matter. A: Segment L7 of the cat spinal cord (Rexed, 1952). B: Segment L5 of the rat spinal cord (Paxinos and Watson, 1982).
to suggest that lamina I is the major termination site for Aδ primary afferents (Beal and Fox, 1976; Beal and Bicknell, 1981; Fyffe, 1984). A particular group of neurons in lamina I receives inputs from nociceptive-specific C fibers (McMahon et al., 1984; Sugiura et al., 1986) and responds only to heavy pressure, pinch, noxious chemicals and to heat above 45° (Perl, 1985; Willis, 1985). In terms of the functional properties, lamina I neurons can be classified into three types: nociceptive specific neurons (NS), wide-dynamic range neurons (WDR), which respond to both nociceptors and low threshold mechanoreceptors, and thermoreceptors. In fact, thermoreceptive neurons concentrate in lamina I (Price and Dubner, 1977). At least ten neuropeptides have been found to localize in lamina I neurons, including SP, DYN, ENK, NKA and CCK (Willis, 1991).

**Lamina II**

The lamina II is just ventral to lamina I and is also termed “substantia gelatinosa” (Cervero and Iggo, 1980), for it is a grossly distinguishable layer of the superficial dorsal horn. The gelatinous appearance of this area is due to the concentration of small neurons and their processes plus a striking absence of myelinated axons (Rexed, 1952, 1954). Based on the dendritic arborizations and axonal projections of its neurons, this lamina is subdivided into two regions, an outer region (lamina IIo: 30-40 μm thick) and an inner region (lamina IIi, 40-50 μm thick) (Rexed, 1952; Brown, 1981). There are two types of neurons found in lamina II. The larger, stalked cells (16-22 μm) are mostly found in the lamina IIo (Bennett et al., 1980). Their dendritic trees are mostly confined in lamina II, projecting ventrally, although dorsally in some cases to lamina I (Sugiura, 1975; Brown, 1981). The other type of cells is smaller islet cells (5-10 μm in diameter) with longitudinally oriented dendritic
trees, spreading throughout lamina II and III. They are found mainly in lamina III (Scheibel and Scheibel, 1968; Sugiura, 1975). Functionally, the lamina IIo contains either NS or WDR neurons, whereas neurons in the inner part of lamina II respond only to innocuous mechanical stimuli (Brown, 1981). The primary input to lamina II is from unmyelinated C fibers and smaller myelinated Aδ fibers. There are more than fourteen different peptides found in the lamina II, including SP, NKA, VAS, OXY, CCK, VIP, ENK, NPY, DYN.

Laminae I and II together make up the superficial dorsal horn. The nociceptive nature of the unmyelinated (C) fibers and small myelinated (Aδ) fibers from skin, muscle, and viscera terminating mainly in this area makes it an important site for transmission and modulation of nociceptive information.

*Lamina III*

Lamina III forms a broad band across the dorsal horn, medially limited by white matter and laterally by substantia gelatinosa. This lamina is distinguished from lamina II in having slightly larger, spindle-shaped neurons, oriented vertically to the surface of the lamina (Rexed, 1952), and in having a neuropil that contains myelinated axons. Two types of cells have been found in lamina III (Willis, 1991), postsynaptic dorsal column cells and spinocervical tract cells. The postsynaptic dorsal column cells are characterized by dorsal dendrites that penetrate laminae I and II, whereas the dendrites of spinocervical tract cells do not. The unidentified neurons in this lamina are of various types. Axons of postsynaptic dorsal column cells take part in the propriospinal pathways (Szentagothai, 1964; Scheibel and Scheibel, 1968). These axons travel widely throughout the gray matter of the dorsal horn before passing into the white matter (Matsushita, 1969, 1970). Upon returning to
the gray matter, the axons project mainly to laminae I and II (Szentagothai, 1964). The dendritic pattern of lamina III cells is relatively complex. The primary afferent input into lamina III is derived from the flame-shaped arbors which have recently shown to carry information from hair follicles (Ralston, 1984). Other types of coarse primary afferents that enter lamina III arise from Pacinian corpuscles (Maxwell et al., 1984a; Ralston, 1984). Functionally, the lamina III neurons are driven by low-threshold mechanoreceptive fibers (Aβ) and are regarded as inhibitory interneurons. The peptides are less abundant in this area: SP, ENK, NT and TRH.

_Lamina IV_

Lamina IV is a relatively thick layer that extends across the dorsal horn. Its medial border is the white matter of the dorsal column, and its lateral border is the ventral bend of laminae I-III. The neurons in this layer are of various sizes, ranging from very small (≈8×11μm) to quite large (≈35×45μm). Although outnumbered by the smaller cells, the large, star-shaped neurons are so prominent that it seems this is a layer of large cells (Rexed, 1952). The dendrites of the large cells, dorsally oriented, penetrate into substantia gelatinosa (Szentagothai, 1964). Axons of lamina IV neurons project cranially via spinothalamic and spinocervical pathways, or contribute to propriospinal pathways (Szentagothai, 1964; Willis and Coggeshall, 1978). Primary afferent input to the lamina IV cells converges on their dorsal dendrites located in superficial laminae. The neuropeptide content in this layer is similar to that in lamina III (Willis, 1991).
Lamina V

Lamina V forms as a thick band across the narrowest part of the dorsal horn, which is known as the neck of the dorsal horn. The medial part of this lamina is bordered distinctly by the dorsal funiculus, while the lateral border is obscure because of the many bundles of myelinated fibers coursing longitudinally through this area (Willis 1991). The cells in lamina V are even more varied in sizes and shape that those in lamina IV. The axonal projections of lamina V cells appear to go to the thalamus, the dorsal column nuclei, the lateral cervical nucleus and various local destinations in the cord. The dendritic organization of lamina V cells is similar to that of lamina IV cells, except the general orientation of the trees changes to vertical, in contrast to the generally longitudinal orientation in lamina IV. Lamina V is a major projection area of the viscera, group IV fibers from the muscle and Aδ fibers from contralateral skin nociceptors (Light and Perl, 1979; Cervero and Connell, 1984). At least eight different neuropeptides have been found in lamina V: SP, CCK, SRIF, ENK, DYN, NPY, CRF and GAL.

Excitatory neurotransmission in the spinal dorsal horn

It is now accepted that activation of primary afferent fibers initiates the release of two major types of chemical compounds in the spinal DH, dicarboxylic amino acids (glutamate and aspartate) and tachykinins (substance P and neurokinin A). Both of the two classes of compounds are heavily distributed in the dorsal root ganglion (DRG) cells and in spinal DH (Henley et al, 1993). Glutamate and aspartate are believed to mediate the fast excitatory synaptic transmission (Mayer and Westbrook, 1987; Gerber et al., 1989; Yoshimura and Nishi, 1993) whereas substance P (SP) and
neurokinin A (NKA) are involved in the slow excitatory neurotransmission (Urban and Randic, 1984).

The fast excitatory postsynaptic potentials (EPSP) mediated by glutamate is thought to arise from the binding to and activation of glutamate receptors. There are two types of glutamate receptors, ionotropic receptors that gate cation channels permeable to Na\(^+\), Ca\(^{2+}\) and K\(^+\) upon activation (Mayer and Westbrook, 1987) and metabotropic receptors that are coupled to G protein and intracellular second messenger systems (Baskys, 1992). The ionotropic glutamate receptors have been separated into NMDA (N-methyl-D-aspartate) and non-NMDA receptors. The NMDA receptor is a clearly distinct entity and can be pharmacologically distinguished from the non-NMDA receptors by an antagonist D-2-amino-5-phosphonovalerate (APV). There are two kinds of non-NMDA receptors, characterized by their selective agonists, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (KA) (Mayer and Westbrook, 1987; Watkins et al., 1990).

The fast EPSPs mediated by both NMDA and non-NMDA receptors have been implicated in the transmission or modulation of information in various parts of the CNS including the spinal cord (Mayer and Westbrook, 1987; Randic et al., 1993). It is believed that the activation of NMDA receptor is necessary in the long term potentiation (LTP) of excitatory synaptic responses in various regions including the CA1 region of the hippocampus (Malenka and Nicoll, 1993). The AMPA receptor-mediated synaptic transmission has been shown to undergo long-term depression (LTD) in the cerebellum, hippocampus and cortex (Linden and Connor, 1991; Budek and Bear, 1992). In addition, the excitatory synaptic responses mediated by NMDA and non-NMDA receptors can be modulated by various intracellular and extracellular
compounds. Tachykinins (Rusin et al., 1991), opioids (Kolaj et al., 1994; Cerne et al., 1995) and protein kinases (Cerne et al., 1993) are among the group of compounds shown to affect the excitatory responses of the spinal DH neurons. Of particular interest here are the findings that activation of κ or μ-opioid receptors modulates NMDA (Cerne et al., 1995) and/or non-NMDA (Kolaj et al., 1995) receptor-mediated responses in the isolated spinal DH neurons.

**γ-Aminobutyric acid and its receptors**

The γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS). Synaptic inhibition in the mammalian brain results largely from the actions of the amino acid on its receptors (Mody et al., 1994; Qian and Dowling, 1994). In the cerebral cortex, it is estimated about 17% of the synapses are GABAergic (Halasy and Somogyi, 1993). GABA released from presynaptic nerve terminal binds to either GABAA or GABAC receptor, which gate a chloride channel (Qian and Dowling, 1993; Macdonald and Olsen, 1994), or GABAB receptor, which is coupled to G proteins (Mody et al., 1994). Activation of GABA_A receptors opens Cl\(^{-}\) channels and produces a fast inhibitory postsynaptic potential (IPSP). A “slow” inhibition which usually follows the fast IPSP is believed to result from the activation of GABA_B receptors. There are two membrane effects of GABA_B receptor activation which are associated with the slow IPSP: increase in K\(^{+}\) conductance and decrease in Ca\(^{2+}\) conductance. While it was previously believed that the two effects were related, later studies indicate they are not (Dutar and Nicoll, 1988; Bowery, 1989; Wang and Dun, 1990). In fact, the reduction in Ca\(^{2+}\) conductance, produced in some sensory neurons giving rise to Aδ and C primary afferents, was not attenuated by inhibitors
of K⁺ conductance (Bowery, 1989).

The GABA\textsubscript{A} receptor belongs to the ligand-gated ion channel superfamily (Swope et al., 1992). It is thought to have two binding sites for GABA, high (nM) and low (\mu M) affinity sites (Sakmann et al., 1983; Macdonald and Olsen, 1994). Based on a variety of molecular studies, purified GABA\textsubscript{A} receptors consist of members from five subunit families: α, β, γ, δ and ρ (Olsen and Tobin, 1990; Macdonald and Olsen, 1994). About 30-40% sequence identity is found among the subunit families. There is 20-30% of sequence homology among all the subunits and other gene products of the ligand-gated receptor superfamily (Schofield et al., 1987; Olsen and Tobin, 1990; Swope et al., 1992). Most of the subunit families have multiple subtypes (α1-6, β1-4, γ1-3, δ and ρ1-2). All of the sequences within each subunit family have about 70-80% of homology. Each GABA\textsubscript{A} receptor subunit cDNA encodes for polypeptides of about 50 kDa, with putative N-glycosylated sites, and four α-helical hydrophobic membrane-spanning regions (Schofield et al., 1987; Olsen and Tobin, 1990). The cytoplasmic region of highly variable sequence between the third and fourth membrane-spanning regions is believed to mediate regulatory mechanisms such as phosphorylation.

The current model of the GABA\textsubscript{A} receptor-ion channel complex is a heteropentameric glycoprotein of about 275 kDa, composed of the combinations of multiple subunits. The subunits form a quasisymmetric structure around the channel. Studies using the technique of heterologous expression of GABA\textsubscript{A} receptor subunit mRNAs in \textit{Xenopus} oocytes and mammalian cells have revealed that functional receptors are formed from monomeric, dimeric, and trimeric combinations (Schofield et al., 1987; Blair et al., 1987; Sigel et al., 1990; Angelotti et al., 1993a). In contrast, the expres-
sion of α1, β2, and γ2S subtypes in HEK 293 fibroblasts has produced only three functional receptors: α1β2, α1γ2, and α1β2γ2S (Verdoorn et al., 1990). The α1β2 receptors were potently blocked by Zn^{2+}, but α1β2γ2S receptors were insensitive to the Zn^{2+}. In addition to its sensitivity to Zn^{2+}, GABA_A receptors have binding sites to the clinically important drugs such as convulsant picrotoxin and depressants benzodiazepines, barbiturates, and the anesthetic steroids (Olsen et al., 1986; Olsen, 1981, 1987). The membrane current responses mediated by GABA_A receptors are always reduced by picrotoxin and generally enhanced by the depressants. It is believed that a majority of the drugs exert their effects through allosterically modulating the binding of GABA (Olsen, 1981, 1987; Ticku, 1991).

GABA_A channel function may be modified by treatment with compounds that change the level of protein phosphorylation. Consensus sequences for cAMP-dependent kinase (PKA), Ca^{2+}-phospholipid-dependent protein kinase (PKC) and protein tyrosine kinase (PTK) have been identified on several subunit subtypes (Moss et al., 1992; Swope et al., 1992; Macdonald and Olsen, 1994). All of the β subunits are substrates for PKA and PKC (Schofield et al., 1987; Ymer, et al., 1989). Only two α subunit subtypes (α4 and α6) have been shown as PKA and PKC substrates (Macdonald and Olsen, 1994). There is good evidence that the GABA_A receptor is also functionally regulated by these kinases. The GABA_A receptor-mediated responses in most cell types, except in cerebellar Purkinje cells (Sessler et al., 1989), were reduced by direct application of catalytic subunit of PKA (cPKA) or cAMP analogues (Porter et al., 1990; Moss et al., 1992). Activation of PKC has also been shown to down-regulate GABA currents in oocytes or fibroblasts expressing recombinant GABA_A receptors (Sigel et al., 1991; Krishek et al., 1994). Furthermore, it
appears that PKC selectively inhibits the fraction of GABA<sub>A</sub> channel flux that was not desensitized by prolonged exposure of agonist (Leidenheimer et al., 1992). It has to be mentioned here that almost all of the studies involving the phosphorylation of GABA<sub>A</sub> receptor were done in vitro, the receptor channel has not been demonstrated to be phosphorylated in vivo. In addition to its possible modulation by PKA, PKC and PTK, recent work demonstrated that GABA<sub>A</sub> receptor subunits also possesses phosphorylation sites for Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM-KII) and cGMP-dependent protein kinase (McDonald and Moss, 1994). The functional significance of this observation, however, is not completely understood.

**GABAergic system in the superficial spinal dorsal horn**

It has been revealed, by immunocytochemical reactions for GABA or glutamate decarboxylase (GAD), the GABA synthesizing enzyme, in association with Golgi staining, that about 24-33% of interneurons are GABAergic in rat spinal laminae I-III (McLaughlin et al., 1975; Barber et al., 1978, 1982; Todd and McKenzie, 1989). A detailed analysis of synaptic endings stained immunocytochemically for GABA has demonstrated numerous GABAergic presynaptic dendrites in the superficial laminae of the primate dorsal horn (Carlton and Hay, 1990). A combined Golgi and immunocytochemical study indicated that a large proportion of GABAergic neurons in lamina III was oriented along the rostrocaudal axis and occupied the lamina III, with some extension into lamina IV and the ventral half of the lamina II (Powell and Todd, 1992). GABAergic islet cells are a source of presynaptic dendrites in the lamina II of rat (Spike and Todd, 1992). In addition to its presence in interneurons in laminae I-III, GABA is also contained in the axons of the dorsal and lateral funiculus, tract
of Lissauer (Hunt et al., 1981; Carlton and Hayes, 1990), as well as in the neurons of the rostral ventral medulla that project to the spinal cord (Reichling and Basbaum, 1990).

There is a particular interest in the synaptic arrangements of GABA elements since the information will help to explain the pre- and/or postsynaptic inhibition that this compound mediates. GAD localization study indicated that GAD terminals are presynaptic to the central terminals in the glomeruli (Barber et al., 1978). Results of both autoradiographic (Ribeiro-DaSilva and Coimbra, 1980) and immunocytochemical studies (Basbaum et al., 1986c, Todd and Lochhead, 1990) also seemed to support the idea that GABAergic inhibition is presynaptic. However, some GAD terminals had been shown to be postsynaptic to primary afferents (Basbaum, 1986c). Moreover, in monkeys primary afferents were found to be presynaptic to a majority of GABA-containing structures (Carlton and Hayes, 1990). A more recent study also showed that GABAergic cells in lamina III have a variety of synapses including some formed by axons which resembled low threshold myelinated mechanoreceptive primary afferents (Powell and Todd, 1992). It was suggested that these inhibitory neurons that contain GABA and glycine selectively regulate the transmission of the information from low threshold mechanoreceptive primary afferents to other dorsal horn neurons. Both GABA<sub>A</sub> and GABA<sub>B</sub> receptor types have been found in the spinal dorsal horn (Bowery et al., 1987). While GABA<sub>B</sub> sites are localized to laminae II and III, the GABA<sub>A</sub> sites, although numerous, are not well localized (Willis and Coggeshall, 1991). GABA<sub>A</sub> receptors are believed to be situated mainly on the postsynaptic membrane (Bowery et al., 1987; Willis and Coggeshall, 1991). GABA<sub>B</sub> receptors, on the other hand, are thought to be present predominantly, but not ex-
clusively, in the presynaptic membrane (Price et al., 1984). In addition, GABA\textsubscript{B} receptors have been demonstrated on a proportion of primary sensory B neurons (Price et al., 1984).

Functionally, there is good evidence to implicate GABA and glycine in the postsynaptic inhibitory actions in dorsal horn interneurons. Iontophoresis of GABA and glycine resulted in the inhibition of the activity of dorsal horn neurons (Curtis et al., 1968, 1977; Werman et al., 1968), including those in substantia gelatinosa (Zieglgansberger and Sutor, 1983). The inhibitory action of GABA could be blocked by GABA antagonists, picrotoxin and bicuculline (Curtis et al., 1969, 1971; Game and Lodge, 1975). GABA and glycine were also shown to mediate the IPSPs in dorsal horn interneurons, produced from stimulation of primary afferent fibers (Game and Lodge, 1975; Rudomin et al., 1990). The involvement of GABA in presynaptic inhibition is supported by numerous studies as well. These include: (1) primary afferent depolarization (PAD) is at least partially blocked by picrotoxin and bicuculline (Gallagher et al., 1978; Rudomin et al., 1990); (2) PAD is increased when GABA-transaminase, a GABA degrading enzyme, is inhibited (Davidoff et al., 1973); (3) Group Ia primary afferent fibers are depolarized by iontophoretical application of GABA (Curtis and Lodge, 1982); and (4) GABA\textsubscript{B} receptor-specific agonist (-)baclofen reduced the dorsal root stimulation-evoked release of glutamate and aspartate (Kangrga et al., 1991).

However, the physiological implications of these GABAergic actions in the spinal DH, observed in vitro or in vivo, are more complex, even conflicting in some cases. There are studies, indicating that the GABAergic actions contribute directly to the inhibition of primary sensory information. Both GABA and muscimol produced
the bicuculline-sensitive inhibition of C-fiber-mediated responses in various isolated spinal preparations (Otsuka and Yanagisawa, 1990). The intrathecally administered picrotoxin and bicuculline attenuated the pentobarbital-produced inhibition of a spinal reflex (Stein et al. 1987). In a recent study using spinal cord stimulation, an established treatment for certain chronic pain syndromes resistant to conventional therapeutic procedures, a significant release of GABA was found in the dorsal horn (Linderoth et al., 1993). These data seem to confirm the inhibitory nature of GABA actions on the transmission of sensory information in spinal DH. However, in some other studies, GABAergic actions seem to have an excitatory effect. Bicuculline, the GABA$_A$ receptor antagonist, has been found to abolish the responses of spinal dorsal horn to noxious heating or innocuous skin brushing (Sandkuhler et al., 1989). In addition, the excitatory response to input from a single group II hair afferent (A$\beta$) was attenuated by administration of bicuculline (De-Koninck and Henry, 1994).

**Opioid system in the spinal dorsal horn**

Due to their role in the pain-relieving treatment and their modulatory effects on sensory information processing in general, exogenous opiate and endogenous opioid pathways in the CNS are among the fields of neuroscience that have been vigorously studied. The endogenous opioid peptides derived from proopiomelanocortin, pre-proenkephalin and pre-prodynorphin (Simon, 1991) are found in the spinal DH. Leu- and met-enkephalin and dynorphin (Hunt et al., 1980; Glazer and Basbaum, 1981) are present in the primary afferent fibers and DH neurons. Specifically, enkephalin-containing neurons are distributed mainly in lamina I and II (Glazer and Basbaum, 1981; Bennett et al., 1982). Some enkephalin-containing terminals belong to the ax-
ons from ventral medulla (Millhorn et al., 1987). Dynorphin-containing interneurons are found in lamina I, II and V (Cho and Basbaum, 1988; Carlton and Hayes, 1989).

Met-enkephalin has been shown to be released from the spinal cord in response to stimulation of afferent fibers and to perfusion with K+ or substance P (SP) (Yaksh and Elde, 1981). Stimulation of C fibers, but not A fibers, in a peripheral nerve caused the release of dynorphin A in lamina I of the dorsal horn (Hutchison et al., 1990). It is believed that the opioid peptides exert their effects largely through binding to their receptors. There are three major and best studied types of opioid receptors, μ, δ, and κ (Simon, 1991), although δ and σ opioid receptors have also been suggested. The molecular biology studies demonstrated that they are members of the superfamily of seven-transmembrane spanning receptors, and share a high degree amino acid sequence similarity, with about 50% of the residues being identical (Reisine and Bell, 1993). Almost all of the opioid receptors are coupled to GTP-binding proteins (G proteins) (Childers, 1993). More specifically, all three major types of the receptors have been shown to couple mainly to Gi or Go proteins (Childers, 1993; Cox, 1993), with the exception of a proportion of DRG neurons in which activation of Gs proteins by opioid receptors has been reported (Chen et al., 1988). Consistent with the high distribution of their endogenous agonists in superficial spinal DH, these opioid receptors are found mainly in laminae I-III (Stevens et al., 1991). Autoradiographic studies have revealed μ-opioid binding sites are correspondingly present in lamina I and II (Atweh and Kuhar, 1977; Mack et al., 1984; Morris and Herz, 1987; Arvidsson et al., 1995). Furthermore, Morris and Herz (1987) reported that δ binding sites are restricted to lamina I. The majority of the κ receptors are also concentrated in the superficial laminae (Slater and Patel, 1983; Morris and Hertz, 1987). In regard to
the classification of opioid receptor types, leu- and met-enkephalins are δ receptor agonists with some affinity for μ receptors. Dynorphin A17 is considered as the endogenous ligand for κ receptors. Recent evidence demonstrated existence of subtypes in the opioid receptor families. Whereas μ receptors are separated into μ1 and μ2 subtypes, κ receptors have been divided into κ1, κ2 and κ3 subtypes, and δ receptors are classified into δ1 and δ2 receptors (Simmon, 1991).

Opiates and opioid peptides have been used at both spinal and supraspinal sites to reduce neuronal responsiveness to nociceptive stimuli. Intrathecal administration of opiates or opioids can elicit analgesia at behavioral levels (Duggan and Fleetwood-Walker, 1993; Yaksh, 1993). The majority of the opioid-induced analgesic effects are thought to be mediated by μ and δ receptors. The activation of the receptors induced predominantly inhibitory effects on spinal DH neurons (Hope et al., 1990, Duggan and Fleetwood-Walker, 1993), although excitatory actions have also been reported (Willcockson et al., 1986, Knox and Dickenson, 1987; Rusin and Randić, 1991; Chen and Huang, 1991). The μ-opioid inhibitory actions can be explained by presynaptic inhibition of the release of excitatory neurotransmitters (Kangrga and Randic, 1991; Hori, et al., 1992) or SP (Yaksh et al., 1980), postsynaptic modulation of the sensitivity of the receptors for neurotransmitter such as glutamate (Rusin and Randic, 1991, Kolaj, et al., 1995) and/or modulation of Ca²⁺/K⁺ conductances (North, 1993). There is evidence to support the idea that opioids of μ- and δ-classes may act presynaptically to inhibit the release of SP from the C fibers (see Yaksh, 1993 for review). Activation of μ-opioid receptor by opioid peptide DAGO attenuated the release of glutamate and aspartate from the rat spinal DH slices elicited by activation of low and high threshold primary afferent fibers (Kangrga and Randic, 1991).
These observations are supported by the results of immunoreactive studies (Glazer and Basbaum, 1981; Arvidsson et al., 1995). There is a good evidence to support the concept that presynaptic inhibition of transmitter release by opioids is due to opioid-induced increase in $K^+$ and/or decrease in $Ca^{2+}$ conductances (Williams and North, 1984; Grudt and Williams, 1993; North, 1993). For example, $Ca^{2+}$ currents are decreased in isolated DRG neurons by $\mu$ opioid agonists DAMGO, PLO17 and morphine (Schroeder, et al., 1991; Moises et al., 1994), in cultured DRG neurons by dynorphin (Gross and MacDonald, 1987) and in guinea pig submucous plexus neurons by met-enkephalin and DPDPE ($\delta$ agonists) (Surprenant et al., 1990). Additional effects of the change in $K^+$ and $Ca^{2+}$ conductances include reduction in the duration of action potentials (MacDonald and Werz, 1986; Crain and Shen, 1990).

**Cyclic AMP-dependent second messenger system in the spinal dorsal horn**

The central unit of the cyclic AMP (cAMP)-dependent second messenger system is adenylate cyclase (Casperson, 1990), which catalyzes the synthesis of the cAMP. The adenylate cyclase is regulated by stimulatory and inhibitory G proteins ($G_s$ and $G_i$), which are usually coupled to membrane receptor proteins (Gilman, 1987; Hepler and Gilman, 1992). Four cAMP molecules activate protein kinase A (PKA), a tetramer with two catalytic and two regulatory subunits, by binding to the two regulatory subunits (Taylor, 1990). The activated PKA has been shown to phosphorylate a variety of intracellular proteins or the intracellular loop(s) of membrane-bound proteins including $GABA_A$ receptor subunits (Macdonald and Olsen, 1994).

The functional consequences of the PKA-mediated phosphorylation of cellular proteins range from the regulation of mRNA synthesis (Comb et al., 1986) to presyn-
naptic modulation of neurotransmitter release (Greengard, 1972; Hu et al., 1993) and postsynaptic modulation of receptor sensitivity (Porter et al., 1990; McVaugh and Waxham, 1992; Cerne et al., 1994). Intracellular application of the catalytic subunit of PKA has been shown to increase the NMDA receptor-mediated responses in the spinal DH neurons (Cerne et al., 1994) and *Xenopus* oocytes injected with rat forebrain NMDA receptor mRNAs (McVaugh and Waxham, 1992). In cultured mouse spinal neurons, PKA decreased GABA<sub>A</sub> receptor-mediated currents (Porter et al., 1990). The presence of a cAMP-dependent second messenger system in the spinal DH has been demonstrated by high levels of binding of forskolin (Worley et al., 1986), an activator of adenylate cyclase. Additional evidence supporting a role for the cAMP second messenger system in the spinal DH includes the findings that extracellularly applied forskolin (Gerber et al., 1989; Rusin et al., 1992), cAMP analogues or cPKA (Cerne et al., 1992) potentiates the excitatory amino acid and synaptic responses in the rat DH neurons.

**Calcium/calmodulin-dependent protein kinase and long-term synaptic plasticity**

The type II calcium/calmodulin-dependent protein kinase (CaM-KII) is an enzyme first identified in brain and then discovered to be widespread in nature (Schulman and Lou, 1989). Immunocytochemical and biochemical studies have demonstrated the presence of CaM-KII in various areas of the CNS including spinal DH (Basbaum and Kennedy, 1986; Benson et al., 1992). In one brain region, the hippocampus, CaM-KII makes up a remarkable 2% of total cellular proteins (Kennedy, et al., 1987; Benson et al., 1992). Extensive studies of the multifunctional CaM-KII
have implicated the enzyme in various forms of neuronal plasticity and memory.

The brain CaM-KII exists as a multimeric complex (650kDa) composed of various numbers of 50kD (α) and 58-60 kD (β/β') subunits (Bennett and Kennedy, 1987; Lin, et al. 1987; Schulman and Lou, 1989). The two types of subunits are expressed from related but different genes and both appear to have enzymatic activity. When the CaM-KII is activated in vitro by Ca^{2+}/calmodulin, it can phosphorylate several brain proteins. It is, at the same time, autophosphorylated at the threonine residue that is next to the calmodulin binding domain (Schworer et al., 1988, Thiel et al., 1988). The autophosphorylation produces a Ca^{2+}-independent kinase activity that persists until the dephosphorylation of the threonine by protein phosphatases (Thiel et al., 1988). CaM-KII is thought to be involved in the presynaptic regulation of the release of neurotransmitters. This is supported by the finding that CaM-KII phosphorylates synapsin I, a protein associated with synaptic vesicles, and decreases its attachment to the vesicle membrane (Nestler and Greengard, 1983). Microinjection of this kinase into presynaptic terminals enhanced the neurotransmitter release at squid giant synapse (Llinas et al., 1985).

The efficacy of synaptic transmission in the CNS is not constant, and is subject to various forms of modulation (Kennedy, 1989; Zucker, 1989). Two of these forms, long term potentiation (LTP) and long-term depression (LTD) of excitatory neurotransmission, are of great interest due to their possible relevance to memory and learning. Both forms of the synaptic plasticity have been shown in various area of brain region (Linden et al., 1991; Malenka and Nicoll, 1993) and the spinal cord (Randic et al., 1993).

LTP is defined as enhancement of postsynaptic potentials lasting for hours to
weeks, which is induced by relatively brief (1s or less), high frequency stimulation (Bliss and Lomo, 1973). LTP is thought to have two phases: induction and maintenance. There is a rapid growth in the knowledge of LTP in the last few years. The most extensively investigated form of LTP is one of the Schaffer collateral-CA1 pathway in the hippocampus, which is believed to be important in spatial learning (Bliss and Collingridge, 1993). It has been suggested that the potentiation of postsynaptic response is mainly due to the increase in presynaptic glutamate release (Malinow and Tsien, 1990). More recent data, however, support a significant role of increased postsynaptic responsiveness (Kullmann and Nicoll, 1992). Several studies indicated that at least in the CA1 region of the hippocampus the activation of NMDA receptors (Collingridge et al., 1983, Bliss and Collingridge, 1993) and elevated [Ca^{2+}]_i (Lynch et al., 1983) in the postsynaptic cells are necessary for the LTP induction.

There is compelling evidence to support the involvement of phosphorylation of NMDA and/or non-NMDA receptors in LTP. Activation of both PKC and CaM-KII are implicated in the induction of LTP since activators and inhibitors of the kinases can enhance and block the LTP induction (see Bliss and Collingridge, 1993 for review). Consistent with this line of evidence are the reports that support a role for CaM-KII and/or PKC in the phosphorylation of NMDA and AMPA receptors (Chen and Huang, 1991; McGlade-McCulloh et al., 1993; Kolaj et al., 1994 and Tan et al., 1994) and observations that in the maintenance phase of LTP, both NMDA and AMPA currents are enhanced (see Madison et al., 1991 for review). In behavioral studies, a deficiency in both spatial learning and LTP were reported in mutant mice that do not express the α-subunit of CaM-KII (Silva et al., 1992). In addition, intraventricular infusion of NMDA receptor antagonist AP5 causes a selective impairment
of space learning and LTP (Morris et al., 1986).

In contrast to the extensive studies of LTP, the phenomenon of LTD of excitatory synaptic transmission was not as actively investigated until recently. LTD is termed heterosynaptic or homosynaptic depending on whether the synapse under study needs to be activated during an induction protocol. It has been reported that prolonged (5-15 min), low-frequency (1 Hz) or high-frequency afferent stimulation lead to a stable LTD in the CA1 region of hippocampus (Dudek and Bear, 1992) or in spinal DH (Cheng et al., 1995). Like LTP, at least in the hippocampus, the LTD induction requires activation of NMDA receptors (see Malenka and Nicoll, 1993 for review) and the rise in $[\text{Ca}^{2+}]_i$ (Sakurai, 1990 and Mulkey and Malenka, 1992) in the postsynaptic cell. In the cerebellar LTD of AMPA-mediated currents (Linden and Connor, 1991), the activation of PKC through metabotropic quisqualate receptors has also been implicated in its induction. The complete picture regarding the mechanism of LTD induction is still unclear. The current view is that LTD is triggered by prolonged activation of NMDA receptor that is below the threshold for the induction of synaptic potentiation (Dudek and Bear, 1992; Malenka and Nicoll, 1993). The subthreshold activation of NMDA receptors produces the $[\text{Ca}^{2+}]_i$ change, which may be distinct from that required for LTP (Mulkey and Malenka, 1992). These reports indicate that activation of NMDA receptors does not necessarily lead to induction of LTP. It is interesting to note that the maintenance of LTD in the hippocampal CA1 region has been shown to require the activation of protein phosphatases (Mulkey, et al., 1993).

Recently, the interest in LTP and LTD has grown beyond their induction and maintenance. Modulation and regulation of LTP and LTD by opioid peptides and GABAergic actions have been shown. Dynorphin is believed to mediate an inhibitory
action on the induction and expression of LTP in the hippocampus (Wagner et al., 1993; Weisskopf et al., 1993). Both GABA_A and GABA_B receptor-mediated responses were found to play a role in regulating long-term excitatory synaptic plasticity (Wigstrom and Gustafsson, 1983; Davies et al., 1991). The development of hippocampal LTP was facilitated by a reduction in the GABA_A receptor-mediated synaptic transmission (Wigstrom and Gustafsson, 1983), and activation of GABA_B autoreceptors in the presynaptic sites was found also to influence the physiological inhibition (Davies and Collingridge, 1993).

In addition to its modulatory actions on excitatory LTP and/or LTD, the inhibitory synaptic response mediated by GABA_A receptors itself can undergo long-term change in efficacy. The LTD and/or LTP of inhibitory synaptic transmission has been shown in the visual cortex (Komatsu, 1994) and cerebellum (Kano et al., 1992). A long-lasting “rebound potentiation” of inhibitory GABA responses in the Purkinje cells was shown in cerebellum (Kano et al., 1992). This rebound potentiation of inhibitory transmission, together with the LTD of excitatory parallel fibers-Purkinje cell transmission, may be important in explaining the cerebellar learning. The exact mechanism of the long-lasting modulation of inhibitory GABAergic responses is still unknown.
CHAPTER 2. ACTIVATION OF $\mu$-OPIOID RECEPTOR MODULATES GABA$_A$ RECEPTOR-MEDIATED CURRENTS IN ISOLATED SPINAL DORSAL HORN NEURONS

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**Introduction**

Gamma-aminobutyric acid (GABA) has been established as a major inhibitory neurotransmitter in the central nervous system. Ordinarily, GABA activates either GABA$_A$/benzodiazepine receptor complexes or GABA$_C$ receptors, that gate an inward flux of chloride ions, or GABA$_B$ receptors that, via G-protein-mediated mechanism, exert multiple effects on cation channels (Sivilotti and Nistri, 1991; Qian and Dowling, 1993). Immunocytochemical studies have revealed that, in rat spinal cord, GABA is present in cell bodies and axonal terminals of 24-33% of neurons in laminae I-III of the spinal dorsal horn (DH) (Todd and McKenzie, 1989), and GABA$_A$ receptor subunit mRNAs are expressed throughout the Rexed's laminae (Persohn et al., 1991). There is good evidence suggesting that GABA, acting at GABA$_A$ and/or GABA$_B$ receptors, is involved in both presynaptic inhibition of primary afferents and

Intrathecal administration of opioid peptides has been found to induce behavioral analgesia by numerous studies (Yaksh, 1993). Antinociceptive action of opioids in spinal cord is believed to be mediated by μ-, δ- and κ-opioid receptors, which are concentrated heavily in the superficial laminae of DH (Morris and Herz, 1987). Activation of μ-opioid receptors produces predominantly inhibitory effects on spinal neurons (Duggan and Fleetwood-Walker, 1993), although excitatory effects were also reported (Chen and Huang, 1991; Rusin and Randic, 1991; Willis and Coggeshall, 1991. The actions of μ-receptor agonists can be explained by presynaptic inhibition of neurotransmitter release, and/or postsynaptic modulation of glutamate receptor sensitivity and Ca²⁺/K⁺ conductances (North, 1993). Presynaptic inhibition of glutamate release by μ-receptor agonists (Kangrga and Randic, 1991) has been shown to reduce the excitatory postsynaptic potentials in the spinal DH neurons (Hori et al, 1992). A postsynaptic mechanism was proposed to explain the enhancement of N-methyl-D-aspartate (NMDA)-activated responses by DAGO (Tyr-D-Ala-Gly-Me-Phe-Gly-ol-enkephalin), a selective μ-opioid agonist, in trigeminal and spinal DH neurons (Chen and Huang, 1991; Rusin and Randic, 1991). Recently, [Met]enkephalin and GABA have been found to colocalize in more than 70% of enkephalin-immunoreactive neurons in rat spinal laminae II and III (Todd et al., 1992). The functional significance of the coexistence remains unclear. To address this question, we studied the effect(s) of DAGO and μ-opioid agonist PLO17 ([N-MePhe³-D-Pro⁴]morphiceptin) on GABA₄ receptor-mediated currents, in acutely dissociated rat spinal DH neurons from laminae I-IV.
Material and Methods

Experiments were carried out on spinal DH neurons acutely isolated from 7-15-day old Sprague-Dawley rats following a published procedure (Murase et al., 1989). The whole-cell voltage-clamp technique was used to record membrane currents of DH neurons at room temperature (22-23°C). DH neurons were continuously perfused (1-2 ml/min) with a solution containing (in mM): NaCl 150, KCl 5, CaCl\(_2\) 0.5, MgCl\(_2\) 1, HEPES 10, D-glucose 10, bovine serum albumin 0.1mg/ml, tetrodotoxin (TTX, 5 x10\(^{-7}\)M), and NaOH to adjust pH to 7.4. The dialysis solution in the patch pipette had the following composition (in mM): KCl 140, NaCl 5, MgCl\(_2\) 1, HEPES 10, EGTA 10, MgATP 2.5 (unless stated otherwise), TRIS base to adjust pH to 7.2. Some of the GABA-induced responses were recorded using the nystatin perforated-patch technique. The perforated-patch recording electrodes were first filled with the internal solution and then back-filled with the same solution containing nystatin (225 µg/ml). The stock solution of nystatin (30-60 mg/ml) was prepared in dimethyl sulfoxide (DMSO) prior to the experiment. GABA (Research Biochemical International, RBI), muscimol (Sigma), DAGO (Cambridge Research Biochemicals, Peninsula Laboratories), PLO17 (American Peptide), naloxone hydrochloride (RBI, Sigma), β-funaltrexamine hydrochloride (RBI), and (-)-bicuculline methiodide (Sigma) were dissolved in the HEPES-buffered external solution and applied to DH neurons through either a Y-tube pressure ejection system (Murase et al., 1989) or an automated DAD-12 pressure application system (Adams and List Associates). Rp-cAMPS (Rp-isomer of adenosine-3', 5'-cyclic monophosphothioate, Calbiochem), Sp-cAMPS (Sp-isomer of cAMPS, L.C. Laboratories) and 8Br-cAMP (Sigma) were applied either extracellularly using the pressure systems or internally by including
them in the pipette solution. Pertussis toxin (PTX, Sigma) was kept in a buffer with high ionic strength (50% glycerol with 50 mM Tris, 10 mM glycine and 0.5 M NaCl, Ph=7.5) before use. The isolated DH neurons used to test the signal transduction mechanism involving GTP-binding protein (G-protein) were incubated with PTX for 5-8 hours (1-2μg/ml) before recording. GABA or muscimol were administered either at 2.5 or 3 min intervals for 4 s or at 0.5-1 min intervals for 500 ms to minimize desensitization. Membrane currents were filtered at 2 kHz and monitored continuously with a List L/M-EPC7 patch-clamp amplifier. pCLAMP software (version 5.5, Axon Instruments) was used on line to record the currents, through a Axolab 1100 system, on a computer. In addition, current and voltage signals were continuously monitored with a Gould-Brush pen recorder. Data are presented as means±S.E.M. Statistical significance between means was determined using either paired or unpaired t-test, and marked by asterisks (* P < 0.05; ** P < 0.01; *** P < 0.001) in graphs.

Results

Whole-cell voltage-clamp recordings were made from a total of 132 DH neurons. Under phase contrast optics, most cells appeared roughly oval or spherical, measuring on average about 15.1x9.9μm. The isolated neurons preserved between 10 to 60 μm of proximal dendrites. Pressure ejection of GABA(2μM, 4s) induced a stable bicuculline-sensitive inward current (peak: 397.0±11.0pA, n=106, Fig.2.1A,B) in DH neurons held at -60mV. When the internal solution containing 2.5mM MgATP and 10mM EGTA was used, the peak GABA responses were relatively stable; the variation was within ±8% of the median amplitude during the course of recording for 30-60 min (Fig.2.1C). Run-down of the current was present in cells recorded
with no MgATP in the pipette solution (to 64.5±6.8% of control, n=3, not shown). These observations are consistent with another report on GABA₄ receptor-mediated responses (Chen et al., 1990). The reversal potential of the current was +3.7 mV (n=4, least square fitting), as expected for the nearly symmetric chloride composition in external and pipette solutions.

To investigate the effect(s) of DAGO on the GABA-induced current, DH neurons were exposed to 0.5 to 500nM DAGO for 2.5 or 5 min, while GABA (2μM, 4s) was applied at 2.5 min intervals, before, during and after the peptide incubation, for 30-60 min or more. The DAGO application did not begin until three stable responses to GABA were recorded. In all the experiments, the perfusing solution contained 5×10⁻⁷M TTX. When DH neurons were clamped to -60mV, the GABA₄-receptor-mediated current was increased by DAGO, in a dose-dependent manner (5nM, by 24.0±8.7%, n=4; 50nM, by 29.3±2.7%, n=12; 500nM, by 51.7±13.6%, n=8, Fig.2.2), in about 62% of the cells examined, and decreased in 25% of cells (5-500nM, by 26.8±4.0%, n=12, not shown). The minimum effective concentration of DAGO producing the enhancing effect was 0.5nM. The potentiation of GABA-induced currents outlasted the period of DAGO application in nearly all the cells affected. Whereas the enhancing effect showed almost full recovery within 10 min after the removal of DAGO in 40% of the cells (50nM, 2.5min, n=5) (Fig.2.3A), in the remainder it persisted for up to 50 min upon DAGO wash-out. Moreover, DAGO by itself either produced a small inward (-2.7±0.4pA, n=14) or outward shift (2.6±0.6pA, n=4) or had no significant effect on the holding current (n=8) in the cells showing the DAGO-induced potentiation of GABA responses. It appears that the DH neurons showing the DAGO-induced increase in GABA responses have sig-
Figure 2.1: Stability of the peak GABA-induced currents in acutely isolated DH neurons. A: The traces show the superimposed inward currents induced in a DH neuron, held at -60mV, by 2 μM GABA alone (trace 1) and together with 10 μM (-)bicuculline methiodide (BIC, 2.5min) (trace 2), a selective GABA<sub>A</sub> receptor antagonist, at time indicated in graph B. 11-day-old-rat. B: Time course of the peak GABA-induced currents before, during and after the application of BIC. C: The averaged time course of the control GABA-activated responses (n=7). TTX (5x10⁻⁷M) was present throughout. Data are presented as mean±S.E.M. V<sub>H</sub>=-60mV. 10-day-old rats.
Figure 2.2: Dose-dependent potentiation of GABA-induced currents by DAGO. A: Time course and concentration-dependent increase in GABA<sub>A</sub> receptor-mediated currents, in a DH neuron held at -60mV. Exposure of the DH neuron to 50 (5min) and 500nM (2.5min) DAGO produced a dose-dependent and reversible increase (by 19.5% and 81.6%, respectively) in the peak GABA-induced current. The whole-cell records of inward currents generated by 2μM GABA, applied at 2.5 min intervals before (trace 1) and after (trace 2) DAGO administration (500nM, 2.5min), are shown in the inset. 10-day-old rat. B: Summary of the dose-dependent potentiation of GABA-induced currents by DAGO (5 nM, by 24±3.7% in 4/10 cells; 50nM, by 29.3±2.7% in 12/22 cells; 500nM, by 51.7±13.6% in 8/9 cells). Vertical bars show ±S.E.M. 7-15-day-old rats. C: The photograph of a DAGO-sensitive DH neuron.
nificantly smaller cell bodies (101.8±5.8μm², n=26) than those showing the decrease (128.3±5.2μm², n=9, P<0.01. unpaired t-test). It would be of interest in a future study to examine whether the electrophysiological properties of the two types of cells differ. In addition to DAGO, another μ-opioid receptor agonist PLO17 was also tested. Extracellular application of PLO17 (0.1-1μM, 4-6 min) to six cells produced a similar enhancing (by 18.5±3.4%, n=4 and n=1 for 1 μM) or depressant (by 20%, n=1) effect on the peak GABA-induced whole-cell currents.

To assess the contribution of membrane resistance change (North, 1993) to the DAGO modulatory effect on GABA responses, we measured the membrane resistance. A 200ms electrical pulse of 20 mV was applied to the DH neurons before and during the administration of DAGO, which revealed an average resistance of 378.8±7.7MΩ in ten cells. Of six DH neurons showing the DAGO potentiating effect on GABA-evoked currents (500nM, by 30.7±4.4%), membrane resistance was decreased in three cells (by 28.3±7.8%) and increased in one cell (by 21.9%) during DAGO application. In the remaining two cells the membrane resistance was not significantly altered.

To further characterize the GABA receptor subtype involved, we next used muscimol, a selective GABA_A receptor agonist. We found that the membrane current induced by muscimol (2μM, 4s) was enhanced by DAGO (5-500nM) in five of six cells examined (by 39.8±9.5%, n=3, Fig.2.3B). This result provides additional support for our finding that DAGO increases the postsynaptic GABA_A receptor sensitivity in a subset of spinal DH neurons.

To determine the subclass of opioid receptor involved in the DAGO potentiation of the GABA-induced current, we next examined the actions of a non-selective opioid antagonist naloxone (NLX) and a μ-receptor-selective antagonist β-funaltrexamine
Figure 2.3: A,B: Time courses of the DAGO-induced enhancement of GABA or muscimol responses in DH neurons. In A, the enhancing effect produced by DAGO (50nM, 2.5min) either almost completely recovered in about 10 min after the the removal of the peptide (filled circle, n=5) or lasted for up to 50 min (open circle, n=6). 7-15-day-old rats. B: The averaged data for three cells, indicating the time course of the potentiating effect of DAGO (5 nM, 6 min) on muscimol-induced currents. Traces in the inset show the inward currents in a DH neuron produced by 2μM muscimol, applied at 3min intervals before (trace 1) and during (trace 2) DAGO application. 7-11-day-old rats. Data are presented as mean±SEM. V_h=-60mV.
The results are summarized in Fig. 2.4. In the presence of 1nM NLX, DAGO (50nM) produced less than 7% change in the magnitude of the GABA-activated current (n=6, Fig. 2.4A, filled circles). In the absence of NLX, on the other hand, GABA-induced responses were significantly increased (20.0±5.3%) by the same concentration of DAGO (n=11, Fig. 2.4A, open circles). As with NLX, 100nM β-FNA, applied prior to and simultaneously with DAGO, was sufficient to substantially block the potentiating effect of the peptide (n=6, Fig. 2.4B). These results strongly suggested the involvement of μ-opioid receptors in the modulation of GABA responses by DAGO.

These results demonstrate that activation of μ-opioid receptor produces an enhancement of GABA_A receptor-mediated currents in the majority of the freshly isolated DH neurons from laminae I-IV of the rat spinal cord. The exact molecular mechanism(s) underlying the modulation of GABA_A receptor-activated conductance by DAGO and PLO17 have yet to be elucidated. DAGO and PLO17 might directly modify the kinetic properties of single GABA_A-receptor channels, but the effect of μ-opioids on GABA_A receptor-activated single channel currents has not been as yet reported. Alternatively, DAGO and PLO17 might activate μ-opioid receptors and hyperpolarize the cell membrane by increasing K^+ and/or decreasing Ca^{2+} conductances via G-protein-dependent mechanisms (North, 1993). The resulting change in the ionic conductances may in turn increase the driving force for the inward GABA-evoked current with the reversal potential of about 0mV and contribute to the μ-opioid potentiating action on GABA responses. However, our preliminary experiments indicated that DAGO produced a decrease in cell membrane resistance and a small outward shift in the membrane holding current in only a fraction of DH neu-
Figure 2.4: Antagonism of the DAGO-induced potentiation of GABA-activated currents in DH neurons by naloxone and β-funaltrexamine (β-FNA). A: In the presence of naloxone (1nM) (onset and removal of naloxone are indicated by the two arrows shown in the graph), the maximum change in peak GABA-activated currents, produced by DAGO (50nM, 2.5min), was less than 7% (filled circles, n=6). In the absence of naloxone, however, DAGO (50nM, 2.5min) evoked statistically significant enhancement of GABA responses (open circles, n=11). 7-15-day-old rats. B: Application of DAGO (50 nM), preceded by and together with administration of 100 nM β-FNA, produced a small increase (8.1±3.0%) in the GABA-induced currents in all the cells tested (n=6). 11-13-day-old rats.
rons showing the potentiating action. Therefore, the $\mu$ opioid-induced potentiation of GABA responses in the whole cell population studied can not be explained by the change in membrane resistance.

Another way in which the GABA-activated conductance may be enhanced by $\mu$-opioids is through G-proteins and intracellular second messenger mechanism(s). Activation of $\mu$-opioid receptors has been shown to inhibit cyclic-AMP (cAMP) formation through a pertussis toxin-sensitive mechanism (Childers, 1993). The predominantly potentiating effect of DAGO and PLO17 on GABA-evoked currents might then occur as a result of $\mu$-opioid receptor-induced, G-protein-mediated inhibition of adenylate cyclase, which in turn may cause a decrease in cyclicAMP-dependent kinase (PKA) activity. The reduction in PKA activity may result in the enhancement of GABA-evoked currents since activated PKA has been found to decrease GABA$_A$ receptor-mediated responses in most cell types including spinal neurons (Porter et al., 1990; Macdonald and Olsen, 1994). The following experiments were designed to test this hypothesis.

The first set of experiments was designed to test the action(s) of analogues of cAMP on the GABA$_A$ receptor-mediated responses in spinal DH neurons. When the membrane permeable analogue of cAMP, 8Br-cAMP, was extracellularly applied to the DH neurons (0.1 mM), GABA responses were depressed (to 75.7±2.2% of control, n=4), and the inhibitory action of 8Br-cAMP persisted at least 25 min after the removal of the cAMP analogue (Fig.2.5A). Another more potent cAMP analogue, Sp-cAMPS, was also tested. Intracellular (0.1-0.5 mM, to 48.3±7.9%, n=3) or extracellular (0.1 mM, to 42.5%, n=1, perforated patch, Fig.2.5B) perfusion with Sp-cAMPS produced a similar inhibitory effect on GABA responses. These results
Figure 2.5: Depressant effect of cAMP analogues 8Br-cAMP (A) or Sp-cAMPS (B), and blockade of μ-opioid-induced potentiating effect by Sp-cAMPS (C,D). A and B: Time courses of the membrane currents in two DH neurons activated by application of 2μM GABA for 4s in A or 5μM for 500ms in B before and after the application of either 8Br-cAMP (A) or Sp-cAMPS (B) (0.1mM, 5min). The perforated-patch technique was used in B. C,D: Blockade of μ-opioid potentiating effect on GABA-induced currents. When Sp-cAMPS (0.1-0.5mM) was included in the pipette solution, PLO17 (1μM) failed to affect the peak GABA responses (compare 1 and 2 in C and D, n=5).
demonstrated that analogues of cAMP that activate the endogenous PKA inhibit GABA$_A$ receptor-activated currents, when applied extracellularly or intracellularly.

If the $\mu$-opioid-mediated potentiation of GABA responses was indeed due to its indirect inhibitory action on the endogenous PKA system, then the potentiating effect on GABA response should be blocked by the analogue of cAMP which activates PKA. The action of PLO17 was hence tested in the presence of Sp-cAMPS. When Sp-cAMPS (500$\mu$M, $n=4$; 100$\mu$M, $n=1$) was included in the pipette solution, extracellular application of PLO17 (1$\mu$M) did not significantly alter the peak GABA responses ($n=5$, Fig.2.5C,D).

The next series of experiments was designed to reproduce the up-regulation of GABA responses by $\mu$-opioid agonists. To mimic the potentiating effect of DAGO and PLO17, the nonhydrolyzable (membrane permeable) analogue of cAMP, Rp-cAMPS, which is a potent competitive inhibitor of PKA, was applied extracellularly (0.1-0.5$\mu$M, 12 min) to the DH neurons while GABA was applied at 1 min intervals before, during and after the application of Rp-cAMPS. A sustained potentiation (by 52.1±7.9%, $n=5$, Fig.2.6A,B) of the peak GABA response was found (perforated-patch, $n=2$), which reached a significant level 3 min following the initiation of Rp-cAMPS incubation. Furthermore, during the incubation of DH neurons with Rp-cAMPS, application of PLO17 (1$\mu$M, $n=1$; 5$\mu$M, $n=2$) did not alter the peak GABA-activated currents ($n=3$, Fig.2.6C,D). This suggests that the enhancing effect of $\mu$-opioids can be occluded by application of Rp-cAMPS.

We then studied the possible involvement of G proteins in the observed effect. Pertussis toxin (PTX), the major protein toxin produced by Bordetella pertussis, has widely been used for the investigation of G protein-related intracellular mechanisms.
Figure 2.6: GABA_A receptor-mediated responses are enhanced by Rp-cAMPS. A: Superimposed traces of the GABA (5μM, 500ms)-induced currents, recorded in a DH neuron before (1) and during (2) application of Rp-cAMPS (0.1-0.5 mM) at times indicated in B. B: Pooled data (n=5, of which two are recorded using perforated patch technique) demonstrating that the peak GABA (5-10μM, 500 ms)-induced currents are potentiated (by 52.1±7.9%) by extracellular application of Rp-cAMPS (0.1-0.5mM, 12 min). C,D: The superimposed traces (C) and time course (D) of the GABA currents in a DH neuron showing that during the Rp-cAMPS (0.1 mM, 20 min) treatment, which induced a potentiation of the currents, PLO17 (1μM, 5min) did not alter the peak GABA responses (compare 2 and 3).
since it selectively blocks the activation of $G_\text{i}$ or $G_\text{o}$ proteins by catalyzing the ADP-ribosylation of a Cys residue (Hepler and Gilman, 1992). We hence tested the effect of $\mu$-opioid agonist PLO17 on GABA currents in neurons that had been incubated with PTX (1-2$\mu$g for 5-8 hours), an inhibitor of $G_\text{i}$ and $G_\text{o}$ proteins (Mousli et al., 1990). In the DH neurons pretreated with PTX, the GABA responses were not significantly altered during the application of PLO17 (1$\mu$M, $n=4$). Following the removal of the opioid, a small decrease in the GABA currents was observed (to 93.4±4.1%, Fig.2.7A,B).

The results presented so far strongly suggest that the $\mu$-opioids might have exerted their enhancing action on GABA-induced current in rat spinal DH neurons through a G protein-coupled cAMP-dependent protein kinase system. However, these experiments designed to validate the steps in the pathway of the hypothesized mechanism of the $\mu$-opioid actions on GABA currents are all done in different populations of cells. Therefore, an attempt was made to mimic and to block the $\mu$-opioid effect in the same cell, as shown in Fig.2.8. In one DH neuron, the application of PLO17 (100 nM) potentiated the GABA response. Following the removal of PLO17, Rp-cAMPS (0.1 mM) mimicked the potentiating effect. However, during the application of Sp-cAMPS (0.1 mM), which produced a reduction in the GABA responses, PLO17 (200 nM) failed to affect the GABA-induced currents.

**Discussion**

The present study demonstrates that $\mu$-opioid receptor agonists DAGO and PLO17 modulate $\mathrm{GABA}_A$ receptor-mediated responses in about 89% of freshly isolated DH neurons from laminae I-IV of the rat spinal cord. DAGO produced a signif-
Figure 2.7: Pertussis toxin (PTX) blocked the potentiating effect of PLO17 on GABA-induced responses. A: Superimposed traces of the GABA-induced currents recorded in a DH neuron, pretreated with PTX (1 µg) for 6.5 hours, before (1) and after (2) application of PLO17 at times indicated in B. B: The averaged time course of the peak GABA currents in the DH neurons pretreated with PTX (1-2 µg for 5-8 hours, n=4).
Figure 2.8: Potentiating effect of PLO17 and Rp-cAMPS and the blockade of the PLO17 action on GABA-induced currents by Sp-cAMPS. A: Superimposed traces of the GABA(5μM, 500ms)-induced currents in a DH neuron recorded before (1,3,5) and after (2,4,6) the extracellular application of PLO17 (0.1μM), Rp-cAMPS (0.1mM) and PLO17 (0.25μM) plus Sp-cAMPS (0.1mM), respectively, at times indicated in B. B: The time course of GABA(5μM, 500ms)-induced currents, in the DH neuron, in the respective presence of PLO17 (0.1μM, 5min), Rp-cAMPS (0.1mM, 3min), and Sp-cAMPS (0.1mM) plus PLO17 (0.25μM, 5min).
icant enhancement of GABA-activated currents in about 65% of the examined cells, when applied in concentrations as low as 0.5nM. Since a similar effect of DAGO was found on the membrane currents induced by muscimol, a GABA\textsubscript{A} receptor-specific agonist, this suggests that it is the current response mediated by GABA\textsubscript{A} receptors that may be modulated by \(\mu\)-opioids. The findings that the potentiating action of \(\mu\)-opioids can be blocked or reduced by naloxone and \(\mu\)-opioid receptor-specific antagonist \(\beta\)-funaltrexamine further suggest that the \(\mu\)-opioid effect is specific, involving activation of \(\mu\)-opioid receptors. Similar modulatory action(s) of \(\mu\)-opioid agonist on postsynaptic GABA\textsubscript{A} receptors have not been reported in the spinal DH previously.

The \(\mu\)-opioid receptor agonist-mediated potentiation of GABA response can be mimicked and occluded with the cAMP analogue which inhibits PKA, and blocked by the cAMP analogue which activates PKA. The observed action of cAMP analogues on GABA-induced responses in the freshly isolated spinal DH neurons is consistent with the predominant down-regulation of the GABA\textsubscript{A} receptor by PKA shown in a variety of cell types, including spinal neurons (Macdonald and Olsen, 1994). For example, PKA has been shown to decrease GABA-induced currents in cultured spinal (Porter et al., 1990) and sympathetic ganglion neurons (Moss et al., 1992) but to increase them in the cerebellar Purkinje cells (Sessler et al., 1989; Kano and Konnerth, 1992). In addition, numerous studies have confirmed that the activation of \(\mu\)-opioid receptors has an inhibitory effect on the intracellular cAMP production (Yu et al., 1990; Childers, 1993). The potentiating effect of \(\mu\) agonists on GABA current was blocked in the DH neurons pretreated with PTX, which inhibits \(G_i\) and \(G_o\) proteins. In agreement with this line of results is the coupling of \(\mu\)-opioid receptors to \(G_i\) and \(G_o\) proteins, shown in diverse types of cells (see Cox, 1993 for review). For example,
opioid effects mediated by $\mu$ receptors in guinea pig ileum (Tucker, 1984) and in rat locus coeruleus (Aghajanian and Wang, 1986) are both prevented by treatment with PTX.

Taken together, our results support the hypothesis that the $\mu$-opioid-induced potentiation of $\text{GABA}_A$ receptor-evoked currents is mediated by activation of $G_i$ or $G_o$ proteins, which in turn inhibit the PKA pathway through a reduction in cAMP production. The inhibition in PKA activity produces the enhancement in $\text{GABA}_A$ responses, which has been observed in our study. Currently, the mechanism involved in the inhibitory effect of $\mu$-opioids on GABA-induced currents, observed in a small proportion of DH neurons, is still unknown.

GABAergic actions at $\text{GABA}_A$ receptors have been implicated in antinociceptive actions in the spinal cord (Otsuka and Yanagisawa, 1990; Stein et al., 1987). Both GABA and muscimol produce the bicuculline-sensitive inhibition of C-fiber-mediated responses in various isolated spinal preparations (Otsuka and Yanagisawa, 1990). $\text{GABA}_A$ receptor antagonists picrotoxin and bicuculline administered intrathecally attenuate the pentobarbital-induced inhibition of a spinal nociceptive reflex (Stein et al., 1991). Our finding that $\text{GABA}_A$ receptor-mediated responses in DH neurons can be enhanced by activation of $\mu$-opioid receptors suggests that opioids might exert their antinociceptive effects at least in part by increasing the sensitivity of postsynaptic $\text{GABA}_A$ receptors in the spinal cord. This finding is consistent with the colocalization of GABA and [Met]enkephalin in 70% of enkephalin-immunoreactive neurons in the spinal laminae II and III (Todd, et al, 1992). It further agrees with a recent behavioral study that shows that the blockade of $\text{GABA}_A$ or $\text{GABA}_B$ receptor-mediated inhibitory transmission in the mouse spinal cord reduces the antinocicep-
tion induced by the intracerebroventricular administration of DPDPE, a δ-prefering analogue of enkephalin (Holmes and Fujimoto, 1994). Our observation, however, contrasts with the predominantly depressant effects of opiate alkaloids and δ-preferring agonist [Leu]enkephalin on GABA-evoked responses in cultured mouse spinal neurons (Barker, 1978; Werz and Macdonald, 1982). The enhancing effect, especially the sustained action, of μ-opioids could contribute to the regulation of the excitability of spinal DH interneurons and the strength of primary afferent neurotransmission, including nociception.

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CHAPTER 3. \(\alpha\)-SUBUNIT OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II ENHANCES \(\gamma\)-AMINOBUTYRIC ACID AND INHIBITORY SYNAPTIC RESPONSES OF RAT NEURONS IN VITRO

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**Introduction**

Gamma-aminobutyric acid (GABA) is recognized as the major inhibitory neurotransmitter in the mammalian central nervous system. Ordinarily, GABA activates either GABA\(_A\)/benzodiazepine receptor complexes or GABA\(_C\) receptors, which gate an inward flux of chloride ions, or the GABA\(_B\) receptor which via a G-protein-mediated mechanism exerts multiple effects on cation channels (Macdonald and Olsen, 1994; Mody et al. 1994). Most of the fast synaptic inhibition in the mammalian brain is accomplished through activation of GABA\(_A\) receptors; the receptors are also the major sites of action of benzodiazepines, barbiturates and anesthetics

¹The experiments involving the intracellular recording in the hippocampal slices were done by G. Cheng; M. Kolaj provided results from four cells concerning calyculin A.
(Mody et al., 1994). Immunocytochemical studies have revealed that in the rat spinal cord, GABA is present in cell bodies and axonal terminals of 24-33% of neurons in laminae I-III of the spinal dorsal horn (DH) (Todd and McKenzie, 1989). There is good evidence suggesting that GABA, acting at GABA$_A$ and/or GABA$_B$ receptors, is involved in both presynaptic inhibition of primary afferents and postsynaptic inhibition of spinal interneurons. GABA is also present in the hippocampus, where it evokes at least three types of postsynaptic inhibitory synaptic potentials (Nicoll et al., 1990).

A major mechanism for regulating the function of ligand-gated ion channels, including GABA$_A$ receptors, is to modify receptor structure covalently via protein phosphorylation (Raymond et al., 1993; Walaas and Greengard, 1991). Protein phosphorylation plays a major role in various aspects of neuronal function (Walaas and Greengard, 1991; Hanson and Schulman, 1992; Raymond et al., 1993), and in the CNS, protein kinases regulated by second messengers such as calcium, cyclic nucleotides, or phospholipids show regional patterns of distribution that may reflect particular functional roles (Walaas and Greengard, 1991). Consensus sites for a variety of protein kinases can be found within the intracellular domains of many GABA$_A$ receptor subunits (Kirkness et al., 1989; Browning et al., 1990). In accordance with these observations phosphorylation by protein kinase A (PKA; Ymer et al., 1989; Swope et al., 1992), protein kinase C (PKC; Whiting et al., 1990; Moss et al. 1992) calcium/calmodulin-dependent protein kinase II (CaM-KII; McDonald and Moss, 1994) and cGMP-dependent protein kinase (McDonald and Moss, 1994) of GABA$_A$ receptor subunits has been demonstrated. In addition, GABA$_A$ receptors may also be substrates for tyrosine kinase (Pritchett et al., 1989; Swope et al., 1992).
Considerable evidence exists for direct regulation of \( \text{GABA}_A \) receptor function by protein phosphorylation (see Macdonald and Olsen, 1994 for review). A basal level of phosphorylation of either the \( \text{GABA}_A \) receptor itself, or a closely associated protein, appears to be necessary to maintain \( \text{GABA}_A \) receptor function (Chen et al., 1990). In addition, \( \text{GABA}_A \) receptors are substrates of a number of different serine/threonine protein kinases that are regulated by distinct second messengers. The \( \alpha \) subunit family and subunits appear to be the major sites of phosphorylation for these kinases (Moss et al., 1992; Raymond et al., 1993; Krishek et al., 1994; McDonald and Moss, 1994). However, the functional effects observed have been complex, and sometimes even contradictory (Sigel and Bauer, 1988; Sessler et al., 1989; Porter et al., 1990; Ticku and Mehta, 1990; Leidenheimer, 1992; Kano and Konerth, 1992; Moss et al., 1992; Krishek et al., 1994; Lin et al., 1994). Different effects are thought to be due to different receptor subunit combinations in the various preparations, or to the indirect effects of phosphorylation on other regulatory proteins.

Protein phosphorylation functions as a reversible signaling system and equally effective dephosphorylation of protein kinase target enzymes is required to terminate the responses and maintain phosphorylation system responsiveness. Several brain phosphatases have been identified and dephosphorylation as a signal transduction mechanism has been established in the regulation of many cellular processes in the CNS (Walaas and Greengard, 1991). Experimental evidence indicates that the amount of GABA-induced chloride currents in acutely dissociated CA1 hippocampal pyramidal cells is determined by competition between a phosphorylation process that maintains and a \( \text{Ca}^{2+} \)-dependent dephosphorylation process that reduces \( \text{GABA}_A \) sensitivity (Stelzer et al., 1988; Chen et al., 1990). Whereas the \( \text{Ca}^{2+} \)/calmodulin-
dependent phosphatase calcineurin appears to be involved in the inhibitory of the 
GABA response (Chen et al., 1990), the kinase involved in phosphorylation and 
maintenance of the GABA response has not yet been identified.

The brain CaM-KII exists as a multimeric, 650 kDa complex composed of dif­
ferent proportions of 50 kDa (α) and 58-60 kDa (β/β') subunits, both types of sub­
unit appear to express enzymatic activity (Bennett and Kennedy, 1987; Lin et al., 
1987). Immunocytochemical (Basbaum and Kennedy, 1986; Benson et al., 1992) 
and biochemical studies (Walaas et al., 1983) have demonstrated the presence of the 
α-subunit of CaM-KII in the spinal dorsal horn (DH) and brain; high activity was 
found particularly in the hippocampus (Benson et al., 1992). CaMII kinase is lo­
calized both pre- and postsynaptically. Presynaptically, the enzyme phosphorylates 
synapsin I and regulates transmitter release (Walaas and Greengard, 1991). Postsy­
naptically, the α-subunit of CaM kinase II is the major postsynaptic density protein 
(Kennedy et al., 1983; Kelly et al., 1984) at glutamatergic synapses. Recent stud­
ies have indicated that glutamate receptors are functionally modulated by CaM-KII 
(McGlade-McCulloh et al., 1993; Kolaj et al., 1994). Of particular interest is the 
role such modulation is thought to play in synaptic plasticity of excitatory synaptic 
transmission (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992). How­
ever, long-term changes in inhibitory synaptic transmission have been studied more 
recently (Kano et al., 1992; Korn et al., 1992; Komatsu and Iwakiri, 1993; Komatsu, 
1994), but the molecular mechanisms and possible role of CaM-KII are not known.

In this work we have used electrophysiological techniques to study the func­
tional consequences of phosphorylation of native DH and CA1 hippocampal pyrami­
dal GABA_A receptors by CaM-KII. We first investigated whether the alpha subunit
of CaM-KII and calyculin A, an inhibitor of protein phosphatases 1 and 2A (Ishihara et al., 1989; Suganuma et al., 1990), can modulate the current responses of acutely isolated rat spinal DH neurons to activation of GABA$_A$ receptors under whole-cell voltage-clamp conditions. In addition, the present study was designed to determine whether CaM-KII could affect synaptically activated GABA$_A$ receptor-mediated responses, as opposed to responses to exogenous application of GABA. Therefore, we examined the effect of intracellular application of the $\alpha$-subunit of CaM-KII on inhibitory postsynaptic potentials (IPSPs) of CA1 hippocampal pyramidal cells, elicited by stimulation of the stratum oriens/alveus border, with the use of conventional intracellular recording technique in the slice preparation.

**Methods**

Single spinal DH neurons in the Rexed's laminae I-IV were isolated acutely from 7-15-day-old Sprague-Dawley rats by the method described elsewhere (Kolaj et al., 1994). The whole cell voltage-clamp technique was used to record membrane currents of isolated DH neurons to $\gamma$-aminobutyric acid (GABA) at room temperature (20-23°C). Currents were filtered with an external eight-pole Bessel filter set at corner frequency 2 kHz (-3 dB), and an Axolab 1100 system with p-CLAMP (version 5.5) software was used on-line to sample current signals at 2 ms/point. The solution perfusing the outside of the cell contained (in mM): 150 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 10 D-glucose, NaOH to adjust pH to 7.4, 0.1 mg/ml bovine serum albumin and 500 nM tetrodotoxin, osmolarity being 300 mosmol. Electrodes were filled with one of three internal solutions (in mM): 1) 140 KCl, 5 NaCl, 1 MgCl$_2$, 10 HEPES, 10
ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 2.5 Mg-ATP, and tris(hydroxy-methyl)aminomethane (Tris) base for pH 7.2, 295 mosmol, 2) 140 CsCl, 10 NaCl, 1 MgCl₂, 10 EGTA, 10 HEPES, and 5 Mg-ATP, pH 7.4, and 3) for perforated-patch recordings electrodes were first filled with (in mM): 140 CsCl, 10 NaCl, 1 MgCl₂, 10 HEPES, 1 EGTA, 5 MgATP, (pH 7.2) and then back-filled with the same solution containing nystatin (225 μg/ml). The stock solution of nystatin (30-60 mg/ml) was prepared in dimethyl sulfoxide (DMSO) prior to the experiment. The final concentration of DMSO never exceeded 0.1% and DMSO itself was found to have no effect on GABA responses. Once filled with an intracellular solution, electrodes had resistances of 5-10 MΩ. Membrane potential was clamped at -60 mV, where no holding current (leak current) was usually observed. Drugs used were: adenosine 5'-0-(3-thiotriphosphate (ATP-γ-S) (Sigma, Boehringer-Mannheim), (-) bicuculline methiodide (Sigma), calyculin A (LC laboratories), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Cambridge Research Biochemicals), D-2-amino-5-phosphonovaleric acid (D-APV, Research Biochemicals International, RBI), GABA (RBI), muscimol (5-aminomethyl-3-hydroxyisoxazole) (Sigma), (RS)-3-amino-2-(4-chlorophenyl)-2-hydroxy-propylsulphonic acid (2-hydroxysaclofen, Tocris-Neuramin) and tetrodotoxin (Sigma). To examine the CaM-KII effect on the GABA-induced current, the GABA response was triggered once every 30 s. GABA, muscimol and calyculin A were administered using a DAD-12 superfusion system and computer interface (Adams and List Associates).

For conventional intracellular recording of IPSPs, hippocampal slices were obtained from Sprague-Dawley rats (15-22 days-old). Briefly, after the animal was anesthetized with ether and decapitated, the hippocampus was quickly dissected out
and sliced transversely in 4°C oxygenated standard saline solution (see below) with a vibratome to yield several 400 μm thick hippocampal slices. The slices were placed in a holding chamber at 34°C where they were maintained for at least 1 hour before transferring to the submerged recording chamber. The perfusing medium contained (in mM): 124 NaCl, 1.9 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, glucose 10, pH 7.4 when equilibrated with 95% O2 and 5% CO2. To study primarily GABA-receptor mediated synaptic response, the perfusate contained in addition, 50 μM CNQX, 100 μM D-APV and 100 μM 2-hydroxysaclofen to block glutamate-dependent excitatory synaptic transmission and GABA$_B$-receptor mediated inhibitory transmission. The IPSPs we recorded have been identified as GABA$_A$-mediated conductances, because they were fully blocked by the GABA$_A$ antagonist bicuculline (10μM), and were strongly dependent on the transmembrane Cl$^-$ gradient. Activation of GABA$_A$ receptors in a nerve cell during IPSP can lead to either a hyperpolarization, little or no change in membrane potential, or to a depolarization, depending on the relationship between the Cl$^-$ reversal potential and the resting membrane potential of the neuron. GABA$_B$-mediated responses were not observed in this study because weak stimulation was used to evoke synaptic potentials, Cs$^+$ was the main ion in the pipette solution, and 2-hydroxysaclofen was always present in perfusing medium. Only neurons that had a stable resting membrane potential more negative than -60 mV, an overshoot of the action potential and stable resistance, were included in the present report. Under visual control a single fiber glass microelectrode filled with 0.5 M CsCl (pH 7.4; DC impedance 100-160 MΩ) was placed in the CA1 pyramidal cell layer (Fig. 2.5A), and neurons were impaled by oscillating the capacity compensation circuit of the amplifier (Axoclamp 2). The recurrent GABA-ergic inhibitory
pathway of CA1 cells was activated by electrical stimulation of the stratum oriens-alveus border with a coaxial stainless steel stimulating electrode (inner and outer electrodes, 25 and 200 μm OD, respectively; Frederick Haer Co.). A pen-recorder in DC mode was used to continuously record membrane potential; neuronal input resistance was measured by passing hyperpolarizing current pulses (0.05-0.2 nA) across the cell membrane and measuring the voltage deflections produced. An Axoclamp 2A amplifier (Axon Instruments) was used to record data; the Digidata 1200 system with pCLAMP (version 5.5 and 6) software (Axon Instruments) was used for data acquisition and analysis. Single shocks at a fixed suprathreshold strength (0.05-0.10 ms pulses, 2-12 V), repeated at 2 min intervals, were given through a stimulation electrode for 30-45 min following the cell penetration. This frequency of stimulation was chosen for sampling data because it did not result in response facilitation or depression. The synaptic waveforms illustrated are individual traces.

The α-subunit of CaM-KII (gift from Dr. T. Soderling, Vollum Institute) expressed in Sf9 cells and purified, was activated by autothiophosphorylation (McGlade-McCulloh et al., 1993). The autothiophosphorylated CaM-KII was maintained on ice and diluted 10-fold (but only 50% v/v for conventional intracellular recording) in the pipette solution just before use. In the slice preparation the enzyme was ejected by passing the steady negative DC current (0.1-0.5 nA) continuously. At all times during the experiment, hyperpolarizing current was passed so that the membrane potentials of the cells were kept at between -70 and -90 mV. This routinely prevented the generation of spikes or other voltage-dependent events that might result during synaptic stimulation. The concentration of CaM-KII within the recorded cell is unknown. However, hyperpolarizing current would be expected to promote the diffusion
of those basic molecules into the cell. For the control, the CaM-KII was heat inactivated (10 min at 100°C) before addition to the autothiophosphorylation reaction, and the activity of this solution when examined was similar to background values. The tip of the patch pipette was filled with internal solution plus active or heat-inactivated CaM-KII. To compare responses between different cells, the amplitude of GABA- or muscimol-induced current and IPSPs at any given time was normalized with respect to the value of the first response. Results are expressed as means±SEM in the text. Statistical significance was determined by comparing experimental values and control (activated CaM-KII versus heat-inactivated CaM-KII; calyculin A versus control).

**Results**

Fifty-seven acutely dissociated spinal DH neurons from laminae I-IV were used to assess the functional consequences of possible phosphorylation/dephosphorylation of GABA_A receptors by the alpha subunit of CaM-KII and calyculin A, respectively. Recordings from DH neurons at a holding potential of -60 mV using whole-cell voltage-clamp yielded decaying inward membrane currents after the rapid pressure application of GABA (Figs.3.1-3.3). These responses were sensitive to inhibition by bicuculline, suggesting that GABA_A receptors are present on DH neurons. In the presence of control internal solution, the GABA-activated currents evoked by rapid pressure applications of 5-20 μM GABA for 200-500 ms (600 mm Hg) generally did not exhibit significant run-down in the peak amplitude for up to 15 min following formation of the whole-cell recording mode when either KCl (88.4%± 7.6 of the initial response of GABA, mean±SEM, n=9, Fig.3.2B) or CsCl (86.3%±11.2, n=4, Fig.3.3B) were used in the pipette solutions.
Figure 3.1: \(\gamma\)-Aminobutyric acid (GABA)-induced currents in isolated spinal DH neurons, recorded using whole-cell perforated-patch (A and B) or conventional patch-clamp technique (A and C), are potentiated by extracellular application of calyculin A, an inhibitor of phosphatases 1 and 2A. A and C: superimposed traces are the inward current responses evoked by 20 \(\mu\)M GABA (applied for 300ms in A, 200ms in C), recorded before and after the extracellular application of calyculin A (100nM, 7.5min) at the times indicated in B and D. B and D: time courses of the peak GABA (20\(\mu\)M)-activated currents recorded prior to, during and following the application of calyculin A. In B, using perforated-patch technique, a significant increase (\(P<0.05\), unpaired \(t\)-test) in the amplitude of the GABA-induced response was observed during and after calyculin A incubation (\(n=4\), photograph of one neuron is shown in the inset), and in D by use of conventional patch-clamp method calyculin A reversed the run-down of GABA (20\(\mu\)M)-evoked current in a DH neuron recorded without MgATP in internal solution. \(V_h=-60\text{mV}\).
Figure 3.2: GABA-induced currents in acutely isolated DH neurons are enhanced by intracellular application of activated CaM-KII included in a KCl intracellular pipette solution. A: The superimposed traces are the inward current responses evoked by 20 μM GABA (250ms), recorded with the intracellular solution containing 200nM activated CaM-KII. B: The averaged time courses of the peak GABA (5-20μM) currents in DH neurons recorded in the presence of activated CaM-KII (filled circles, n=7), or heat-inactivated CaM-KII (denatured CaM-KII; open triangles, n=7), or under control conditions (2.5 mM Mg-ATP; filled squares, n=9). C. Photographes of one DH neuron, sensitive to CaM-KII, taken before (left) and after (right) whole-cell recording.
Enhancement of GABA-induced current by calyculin A

First we used calyculin A, an inhibitor of protein phosphatases 1 and 2A (Ishihara et al., 1989; Suganuma et al., 1990), to increase the activity of endogenous CaM-KII (Walaas and Greengard, 1991; Dosemeci and Reese, 1993) and to examine the effect of this agent on GABA-induced currents of DH neurons. Extracellular application of 100 nM calyculin A for 7.5 min caused a significant increase in the peak amplitude of GABA_A receptor-activated current in a time-dependent manner. A gradual enhancement (at 20 min to 140.7%±17.6, P<0.05 n=4, Fig.3.1B) in the peak GABA-induced current was observed using both the whole-cell perforated-patch technique (Fig.3.1A,B) and the conventional whole-cell patch-clamp recordings (Fig.3.1C,D).

Enhancement of GABA_A receptor-activated current by CaM-KII

The possible involvement of CaM-KII phosphorylation in GABA_A receptor function was addressed by intracellular application of CaM-KII (200 nM) activated by autothiophosphorylation to give about 70% Ca^{2+}-independent activity. Under these conditions (and in the presence of GABA_B receptor antagonist 2-hydroxysaclofen, n=3), a gradual enhancement in the peak amplitude of GABA_A-receptor-induced current in acutely isolated DH neurons was observed, reaching the significant level after 3-4 min following initiation of whole-cell recording (Figs.3.2B,3.3B). When K^+ was the main intracellular cation, GABA responses were potentiated to a somewhat greater extent by CaM-KII (at 15 min to 176.4%±14.2, P<0.001, n=7, Fig.3.2B) compared with responses recorded in the presence of intracellular Cs^+ (at 15 min to 152.1%±14.1, P<0.05, n=7, Fig.3.3B); all the values showing significant difference from heat-inactivated controls. The presence of the enhancement of GABA response by CaM-KII in Cs^+-loaded cells indicates that the effect is not likely to be
Figure 3.3: The enhancement of GABA_A receptor-mediated currents in DH cells by activated CaM-KII included in a CsCl-internal solution. A: The superimposed traces are the inward GABA(20μM, 200ms)-activated currents recorded at 1 and 20 min after the rupture of the patch in a DH neuron tested with activated CaM-KII (200nM). B: Pooled data show the time courses of the GABA-induced currents in DH neurons recorded using CsCl internal solution containing activated CaM-KII (filled circles; n=7), or heat-inactivated CaM-KII (denatured CaM-KII; open triangles, n=4), or control internal solution (control; filled squares, n=4).
mediated through an interaction of the enzyme with K⁺ channels. The specificity of the action of active CaM-KII was ascertained by recording from cells dialyzed with heat-inactivated CaM-KII, subjected to the autothiophosphorylation reaction. As shown in Figs.3.2B,3.3B, intracellular application of heat-inactivated CaM-KII (200 nM) to DH cells slightly reduced the amplitude of the GABA-induced current, when KCl (82.0%±7.5, n=7) was used and did not change GABA response when CsCl (104.9%±12.1, n=4) was used in the pipette solution. These results suggest that the enhancement of GABA response was mediated by the autothiophosphorylated form of CaM-KII.

CaM-KII significantly slowed the desensitization of response to 100 μM GABA (Fig.3.4), calculated as 100 × [(peakIGABA − 5 s off IGABA)/peakIGABA], from 107%±3.7 (control, n=4) to 89.4%±2.4 (CaM-KII, n=4) at 20 min after the start of recording (P<0.05). Heat-inactivated CaM-II did not modify the desensitization phase of the GABA responses (101.2%±8.2, n=3). No detailed analysis of the effect of CaM-KII on the kinetics of desensitization was performed in the present experiments.

To further characterize the GABA receptor subtype involved, we next used muscimol, a selective GABA_A receptor agonist. We found that the membrane current induced by pressure application of muscimol (2μM for 200-500 ms/600 mm Hg) was increased over 15 min following intracellular dialysis of the α-subunit of 200 nM CaM-KII (Fig.3.5) in all cells tested (150.6%±6.3, n=4). This result provides additional support for our finding that CaM-KII increases the postsynaptic GABA_A receptor sensitivity in spinal DH neurons.

To assess the contribution of membrane conductance change to the CaM-KII modulatory effect on GABA response, we measured the membrane resistance. A
Figure 3.4: CaM-KII reduces desensitization of GABA whole-cell current. A and B: Superimposed traces are the inward currents evoked by 100μM GABA (5s) recorded either with KCl-filled microelectrodes (A) or in the presence of CaM-KII (B) at 2 min or 30 min after the start of whole-cell configuration. The traces have been scaled so that their peak amplitudes are the same. Whereas the extent of desensitization increases (68.2% at 2min and 74.2% at 30min) with the KCl internal solution, it decreases (68.5% at 2min and 61.0% at 30min) in the presence of CaM-KII. The percent desensitization was calculated according to: % desensitization = 100 × [(peak IGABA - 5s off IGABA)/peak IGABA]. C: Pooled data for 8 cells illustrating the time courses of the normalized percent desensitization of the GABA-induced currents in the presence (filled circle, CaM-KII, n=4) and absence (filled squares, control, n=4) of CaM-KII.
Figure 3.5: Potentiation of muscimol-induced currents in isolated DH neurons by CaM-KII. A, superimposed traces are inward currents, evoked by 400ms applications of muscimol (2μM), recorded with the CsCl-filled microelectrodes, in a DH neuron tested with active CaM-KII. B, time course of the muscimol-evoked current responses in the DH neuron treated with active CaM-KII. C. The photograph of the cell.
200 ms electrical hyperpolarizing pulse of 30 mV was applied to DH neurons during dialysis with active or heat-inactivated CaM-KII. Of 4 DH neurons showing the enhancement of GABA response with active CaM-KII, membrane resistance was not significantly altered in 3 cells (96.9%±6.4).

**Potentiation of GABA$_A$-receptor-mediated synaptic response by CaM-KII in hippocampal CA1 neurons**

Because CaM-KII has been implicated in the development of long-lasting changes in synaptic efficacy in the hippocampus (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992), we next examined whether the sensitivity to CaM-KII of the GABA$_A$ receptors that form functional inhibitory synapses in mammalian central neurons was subject to the same modulation by CaM-KII, as the enhancement of GABA receptors expressed in spinal DH neurons. To address this issue we recorded IPSPs from hippocampal CA1 neurons in a transverse slice preparation (Fig. 3.6A), because it is known that in the hippocampus a fast IPSP evoked by stimulation of the stratum oriens/alveus results from GABA activity on somatic GABA$_A$ receptors. Fast IPSPs generated by stimulation of stratum oriens/alveus are monophasic and depolarizing when recorded with cesium chloride-filled electrodes at resting membrane potential, persist during blockade of excitatory transmission (non-NMDA and NMDA receptors with 50μM CNQX and 100μM D-APV, respectively) and GABA$_B$ responses (with 100μM 2-hydroxysaclofen), and are reversibly blocked by the GABA$_A$ receptor antagonist bicuculline (10μM, Fig. 3.6B). The effect of intracellularly applied alpha subunit of CaM-KII on inhibitory synaptic responses, and the temporal development of the CaM-KII-induced changes, are summarized for 6 cells in Fig. 3.6C. The mean of the normalized IPSP amplitudes increased and attained a value of 204.4%±29.8
Figure 3.6: Long lasting potentiation of recurrent inhibitory synaptic transmission in the CA1 hippocampal neurons after intracellular application of activated CaM-KII. A: The arrangement of electrodes for stimulating and recording. B: Individual inhibitory postsynaptic potentials (IPSPs) recorded intracellularly from a CA1 pyramidal cell in response to electrical stimulation (6.6V, 0.05ms) of oriens/alveus, taken before (trace 1), during (trace 2) and 16 min after (trace 3) washout of bicuculline 10 M at Vm = -86 mV. The IPSPs were recorded after pharmacological blockade of non-NMDA, NMDA- and GABA<sub>B</sub> receptors with CNQX 50μM, D-APV 100μM and 2-hydroxy-saclofen 100μM, respectively. C: Summarized data (mean±SEM) showing the time courses of IPSP amplitudes, expressed as percentage of 1st response (control), in the presence of activated 1μM CaM-KII (filled circle; n = 6) or heat-inactivated CaM-KII (denatured CaM-KII; open circle; n = 8). The traces displayed above the graph are individual IPSPs, taken at 2 and 30 min) of a CA1 neuron recorded with a solution containing activated CaM-KII.
(P<0.05) at 20 min after start of recording in all cells into which active CaM-KII was injected. The potentiation always lasted for the duration of the recording period, and no reversal was observed up to 30 min of recording of IPSPs. No significant change (97.6%±18.3, n=6) in input resistance of somas of CA1 pyramidal cells was observed during dialysis with active CaM-KII when measured at 20 min following the onset of recording. The potentiation of IPSPs observed upon intracellular application of active CaM-KII was not seen when heat-inactivated CaM-KII was used. Thus eight CA1 cells injected with heat-inactivated kinase failed to show a significant IPSP increase (92.0%±11.3) at 20 min of recording (Fig.3.6C). In conclusion, the present results demonstrate that CaM-KII can potentiate synaptically-evoked GABA<sub>A</sub> receptor-mediated responses, but the mechanism by which it occurs is unclear.

**Discussion**

Our results indicate that the activity of GABA<sub>A</sub> receptors expressed in acutely isolated neurons from the superficial spinal DH can be modulated by activated CaM-KII and calyculin A. The intracellular application of the alpha subunit of CaM-KII into DH neurons consistently increased the bicuculline-sensitive currents evoked by GABA, the finding suggesting the role of this kinase in the observed effect. This conclusion is in keeping with the presence of this enzyme in the spinal DH (Basbaum and Kennedy 1986; Benson et al. 1992). However in the DH of the spinal cord and CA1 area of the hippocampus, CaM-KII and glutamic acid decarboxylase (GAD), the synthesizing enzyme of GABA, were found to be expressed in different populations of cells, with no colocalization (Benson et al. 1992).
There is evidence for the direct regulation of GABA_A receptors by CAM-KII. Using purified fusion proteins of the major intracellular domain of murine GABA_A receptor subunits produced in Escherichia coli, a recent study identified specific sites of phosphorylation for CaMKII (McDonald and Moss 1994), the finding suggesting that this kinase may play an important role in regulating GABA_A receptors in vivo. Despite the evidence that CaM-KII may phosphorylate the GABA_A receptor subunits (McDonald and Moss 1994), the modulation of GABA_A receptor-activated current by CaM-KII has not been, until now, reported. Although the precise subunit composition of a single neuronal GABA_A receptor in vivo is unknown, phosphorylation of GABA_A receptor subunits by CaM-KII may have contributed to the modulation of GABA_A receptors of rat DH neurons observed in the present work. However, modulation of the GABA_A-activated currents may not have arisen in its entirety or in part, from the direct phosphorylation of GABA_A receptor protein. Because CaM-KII is a multifunctional enzyme, it can catalyze phosphorylation of a diverse group of proteins in vivo, including cyclic nucleotides, phosphodiesterase, calcineurin, and microtubule-associated proteins (Hanson and Schulman, 1992), which in turn may have modulated the channel activity. In addition to the possible direct effect of CaM-KII, intracellular phosphorylation events may also regulate GABA_A receptors indirectly through effects on other regulatory sites. The GABA_A receptors are known to have a variety of modulatory sites which are sensitive to CNS-acting agents such as benzodiazepines, barbiturates and ethanol.

Although there is some evidence that the endogenous phosphatase involved in GABA_A receptor “run-down” may be Ca^{2+}/calmodulin dependent phosphatase (phosphatase 2B or calcineurin) (Chen et al., 1990), our data also suggest the in-
volvement of phosphatases 1 and 2A in the regulation of the receptor function. In addition, our results suggest that postsynaptic protein phosphatases play a fundamental role in the GABA_A receptor function. Our results show that GABA_A receptors are regulated in the spinal DH neurons by an endogenous protein phosphatase(s) that is identical or related to protein phosphatase(s) 1 and/or 2A. This finding is in keeping with the presence of both of these phosphatases within the central neurons (Walaas and Greengard, 1991).

Of particular interest is the role of functional modulation of GABA_A receptors, as shown in the present study, in synaptic inhibition and plasticity in the mammalian brain and spinal cord. Activity-dependent long-term potentiation (LTP) and long-term depression (LTD) of excitatory synaptic transmission appears to take place in many areas of the brain and spinal cord (Bear and Malenka, 1994; Randic et al., 1993). However, most studies have been performed in hippocampus where strong evidence has been obtained for the involvement of CaM-KII- and PKC-catalyzed protein phosphorylation of the postsynaptic glutamate receptors in the development of LTP (Hu et al., 1987; Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992; McGlade-McCulloch et al., 1993; Tan et al., 1994). Current work suggests that the mechanisms of LTP and LTD may converge at the level of specific phosphoproteins (Bear and Malenka, 1994). Regulation of the phosphorylation state of phosphoproteins can be achieved not only by modifying the activity of specific protein kinases but also by modulation of the serine/threonine protein phosphatases activity (Walaas and Greengard, 1991). The activity-dependent increase of AMPA-receptor mediated-synaptic transmission in CA1 region of hippocampus has been recently reported with calyculin A (Figurov et al., 1993). Moreover, calyculin A (Ishihara et al., 1989; Sug-
anuma et al., 1990) blocks homosynaptic LTD in the CA1 area of the hippocampus (Mulkey et al., 1993).

In addition to the plastic changes in excitatory synaptic transmission several recent studies have shown that GABA-ergic synaptic transmission can also undergo changes in efficacy. Thus LTP and LTD of inhibitory synaptic transmission has recently been demonstrated in rat visual cortex (Komatsu and Iwakiri, 1993; Komatsu, 1994). A long-term potentiation of inhibitory synaptic signals has also been reported in the teleost Mauthner cell (Korn et al., 1992) and cerebellar Purkinje cells (Kano et al., 1992). Conflicting results regarding LTP of inhibition have been obtained in the hippocampal slice (Stelzer et al., 1994). However, at present time little is known about the molecular mechanisms involved in the LTP and LTD of inhibitory transmission in the mammalian brain. Because GABA_A receptors are the major sites of synaptic inhibition in the mammalian brain, the sustained modulation of GABA_A receptor by CaM-KII, as shown in the present study, may be potentially important in explaining the long-lasting change at the inhibitory synapse.

In the present study we obtained results suggesting that the CaM-KII-dependent phosphorylation may modulate the efficacy of inhibitory neurotransmission in the CA1 hippocampal neurons. Intracellular application of the active CaM-KII elicited long-lasting potentiation of inhibitory synaptic transmission, similar to LTP described in the rat visual cortex (Komatsu and Iwakiri, 1993), whereas heat-inactivated kinase failed to do so. Clearly, more experiments must be done in the future to further elucidate the role of CaM-KII in the synaptic inhibition in the spinal DH. Nevertheless, the present findings do provide support for an idea that this kinase is involved in a regulation of sensitivity of postsynaptic GABA_A receptors in spinal DH and CA1.
hippocampal pyramidal neurons. It should be noted, however, that this finding is not consistent with recent neuroanatomical data suggesting that CaM-KII is not involved in GABAergic neurons nor in presynaptic mechanisms within the axon terminals of these neurons (Benson et al., 1992). In conclusion, the present results clearly demonstrate a potentiation by CaM-KII of both the exogenously activated GABA currents of spinal DH neurons and the synaptically activated GABA_A receptor-mediated inhibitory potential in the hippocampal CA1 neurons. This mechanism may contribute to long-term enhancement of inhibitory synaptic responses and may also play a role in other forms of plasticity in the mammalian brain.

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CHAPTER 4. MONTE CARLO SIMULATION OF GABA_A RECEPTOR MEDIATED SYNAPTIC TRANSMISSION

Introduction

Neuronal cells in the central nerve system (CNS) communicate with each other at synapses. Usually, electrical depolarization of the presynaptic nerve terminal initiates an influx of calcium ions and fusion of neurotransmitter-containing vesicles with the presynaptic membrane at the synaptic junction (or cleft). Neurotransmitters, the chemical messengers in the CNS, are then released from the presynaptic membrane. A proportion of the transmitters diffuse across the synaptic cleft, bind to the postsynaptic receptors (which are usually transmembrane proteins) and activate a series of biological processes in the postsynaptic cell. Activation of one type of receptors directly opens ion channels, which are often part of the receptors. The acetylcholin (Ach) receptor, the transmitter at the neuromuscular junction, and γ-aminobutyric acid (GABA) type A receptor (GABAR) are two members of the ligand-gated ion channel superfamily. Upon binding and activation of the Ach or GABA_A receptors, the influx of cations (in the case of Ach) or anions (in the case of GABA) produce the excitatory or inhibitory postsynaptic currents (EPSC or IPSC), respectively, in the postsynaptic cell. The corresponding potentials induced by these currents are named EPSP and IPSP.
Both GABA and GABA\textsubscript{A} receptors are widely distributed in the brain and spinal cord. The GABA\textsubscript{A} receptors are considered the major sites at which inhibitory synaptic action takes place in the CNS. The GABA\textsubscript{A} receptor-mediated inhibitory synaptic transmission is among the most actively investigated subjects due to the clinical importance of the pathway. Benzodiazepams and barbiturates are all found to exert their pharmacological actions by binding to the GABA\textsubscript{A} receptors. Alcohol has also been shown to potentiate GABA\textsubscript{A} receptor-mediated response \textit{in vitro}. However, a lot of information regarding the transmission at the GABA\textsubscript{A} synapse remains unclear. Specifically, the timing between the release of vesicles, the concentration of GABA in the cleft, the number of available receptors and the role of receptor desensitization are among the most controversial subjects concerning the GABA\textsubscript{A} synapse. In this Monte Carlo simulation study of GABA\textsubscript{A}-mediated synaptic response, we will attempt to at least give reasonable predictions for the answers to some of these questions.

Monte Carlo analysis of synaptic transmission was first introduced in 1991 by Salpeter and colleagues to simulate miniature endplate current generation in vertebrate neuromuscular junction. It is an alternative to solving simultaneous differential equations for diffusion of neurotransmitters and their binding/unbinding to receptors. The differential equation approach usually focuses on the rate of change of populations of neurotransmitters and receptors in different states. The Monte Carlo method, on the other hand, partitions the postsynaptic membrane into receptor-containing small patches, and randomly specifies, according predetermined probability distributions, the diffusion and/or binding/unbinding/channel opening of individual neurotransmitters and receptors at the start of each “small” time interval $\Delta t$.

The discussion will first start with the theoretical basis of Monte Carlo analysis,
then relates it to the applications in the simulation of synaptic transmission events: transmitter diffusion, binding/unbinding to receptors and channel opening. Comparisons are made between approaches using the deterministic or stochastic differential equation and Monte Carlo analysis. Four theorems related to the comparison and to the error analysis and a Monte Carlo simulation scheme are proved. Validation of the Monte Carlo method in simulations of diffusion and binding/unbinding is also given. The discussion further focuses on the simulations intended to answer some of the questions of significance in biophysical and electrophysiological studies of GABAAergic synaptic neurotransmission, including the ones mentioned above. Results of the simulation studies are then presented and discussed. Finally, the significance of these results is discussed.

Simulation of Diffusion

Different approaches have been used to simulate diffusion of molecules in aqueous solution. In this section, two of the methods using deterministic differential equations and a Monte Carlo simulation scheme are discussed and compared. A spacial discretization procedure and its error analysis are given.

Deterministic differential equation approach

Diffusion of molecules in aqueous solution has traditionally been modeled using partial differential equations. Crank (1975) has given the solutions of the diffusion equations with different initial conditions in various spacial environments. Let us take a look at the solution to the diffusion equation in one dimensional space $X = x : -\infty < x < +\infty$ and see if it has any connection to the probabilistic simulation
of diffusion presented below. The concentration distribution of molecules released from a point source \(x = 0\) at time \(t = 0\), \(A(x, t)\), can be solved from the following equation:

\[
\frac{\partial A(x, t)}{\partial t} = D \frac{\partial^2 A(x, t)}{\partial x^2},
\]

(4.1)

where \(D\) is the diffusion coefficient, a typical value of \(6 \times 10^{-6} \text{cm}^2/\text{s}\) is usually used in simulation of diffusion in aqueous solution. It can be easily checked that \(A(x, t) = (c/\sqrt{t})\exp(-x^2/4Dt)\) is a trial solution for the equation, where \(c\) is a constant determined by the initial conditions.

**Monte Carlo simulation of diffusion**

In simulation of the diffusion of molecules released at \((x, t) = (0, 0)\), the Monte Carlo analysis specifies a “small” time step \(\Delta t\), and determines, according to a predetermined random distribution, the states of each molecule (i.e. distance and direction of diffusion and new location) at the start of each \(\Delta t\). The commonly used probability density function of the random diffusion length \(L\), which is now non-negative, in a time step \(\Delta t\) is

\[
p(L, \Delta t) = \frac{1}{\sqrt{\pi} D \Delta t} \exp(-L^2/4D\Delta t)
\]

(4.2)

Before starting to discuss the Monte Carlo simulation of diffusion using the probability density function, a theorem given below relates the solution of deterministic diffusion equation (4.1) to the probability density function.

**Theorem 1.** The ratio of the solution \(A(x, t)\) of the two-sided deterministic diffusion equation \(\frac{\partial A(x, t)}{\partial t} = D \frac{\partial^2 A(x, t)}{\partial x^2}\) over the distance accumulation of \(A(x, t)\), \(M(t) = \int_{-\infty}^{\infty} A(x, t)dx\), is equal to one half of the probability density function \(p(L, \Delta t)\) if \(x\)
and \( t \) are replaced by \( L \) and \( \Delta t \) respectively.

**Proof:** As stated above, \( A(x, t) = \left( c/\sqrt{t} \right) \exp(-x^2/4Dt) \) is a trial solution for the deterministic equation, with \( c \) as a constant. Then the accumulation of molecules over distance at time \( t \), labelled \( M(t) \), is

\[
M(t) = \int_{-\infty}^{\infty} A(x, t) \, dx = 2c\sqrt{D} \int_{-\infty}^{\infty} \exp(-\epsilon^2) \, d\epsilon = 2c\sqrt{\pi D}
\]

where \( \epsilon = x/(4Dt)^{\frac{1}{2}} \). Since the total number of molecules released at \( (x, t) = 0 \) is fixed, \( M(t) \) is time-independent as expected. Therefore,

\[
\frac{A(x, t)}{M(t)} = \frac{1}{\sqrt{4\pi Dt}} \exp(-x^2/4Dt) \quad (4.3)
\]

It is clear that the ratio is one half of the probability density function \( p(L, \Delta t) \) given in (3.2) if \( x \) and \( t \) are replaced by \( L \) and \( \Delta t \), respectively. The ratio of \( A(x, t) \) over \( M(t) \) represents the proportion (or percentage) of molecules present at \( (x, t) \) per unit distance, which gives a new meaning to the probability density function. The factor \( 1/2 \) arises because \( A(x, t) \) is the diffusion distribution on \( -\infty < x < +\infty \) and \( p(L, \Delta t) \) is the probability density function on \( 0 \leq L \).

Now, we turn to the formation of a Monte Carlo scheme for diffusion simulation. For a given value of \( \Delta t \) and diffusion constant \( D \), the average distance a molecule moves in any Cartesian coordinate is given by a net diffusion length \( L_d \) defined by

\[
L_d = \int_{0}^{\infty} L p(L, \Delta t) \, dL = \sqrt{\frac{4D\Delta t}{\pi}} \quad (4.4)
\]
The distribution function for $L$ is

$$F(L) = \int_0^L \frac{1}{\sqrt{\pi D\Delta t}} \exp\left(-\frac{l^2}{4D\Delta t}\right)dl$$

$$= \text{erf}\left(\frac{L}{\sqrt{4D\Delta t}}\right)$$  \hspace{1cm} (4.5)$$

where $\text{erf}$ is the error function. To generate the random variable $L$ according to the given density function $p(L, \Delta t)$ is to simply to draw a random number $r$ from the uniform distribution in the unit interval, and take for $L$ that value which satisfies $F(L) = r$. In other words, take $L = F^{-1}(r)$, where $F^{-1}$ is the inverse of the distribution function $F$. (Note that: a. monotonicity of $F(L)$ insures the existence of $F^{-1}(r)$ in $r \in [0, 1]$; b. proof of the procedure can found in Appendix A). Hence,

$$L = F^{-1}(r) = \sqrt{4D\Delta t} \text{ erf}^{-1}(r) = \sqrt{\pi L_d} \text{ erf}^{-1}(r)$$  \hspace{1cm} (4.6)$$

where $\text{erf}^{-1}$ is the inverse error function.

In an actual simulation run, however, a modified version of the scheme will be employed because the number of times this function $L(r)$ gets called is tremendous and to computationally find the $\text{erf}^{-1}(r)$ each time $r$ is generated is very time-consuming. To speed up the simulation procedure, Bartol et al. (1991) partitioned the area under the probability density function $P(L, \Delta t)$ into $n$ bins with equal probability of occurring. Define $L_j$ as the point, on the horizontal axis $L$, for the $jth$ bin, where the vertical line passing through it separates the bin into two sub-bins of equal area. Since the probability of moving a distance of length between $(L - \Delta L)$ and $(L + \Delta L)$ for any value of $L$ is proportional to the area under the density function between $(L - \Delta L)$ and $(L + \Delta L)$, then

$$L_j = \sqrt{\pi L_d} \text{ erf}^{-1}\left(j/n - 0.5/n\right)$$  \hspace{1cm} (4.7)$$
where \( j = 1 \) to \( n \), and they can be computed before simulation.

Therefore, for the simulation of diffusion, instead of generating a uniformly distributed number \( r \) from \([0,1]\), a uniformly distributed integer number \( j \) (from 1 to \( n \)) will be generated to determine, by substituting into (4.7), how far each molecule diffuses in one Cartesian coordinate within \( \Delta t \). The estimation of the error induced by the discretization procedure, which has not been published as far as I know, was conducted and summarized in the following theorem.

Before we proceed to present the theorem, a lemma is given, which will be used in the proof of the theorem.

**Lemma 1.** \( 1 - erf(x) \leq e^{-x^2} \)

*Proof:* Define

\[
f(x) = e^{-x^2} + erf(x) - 1
\]

It can checked that \( f(0) = 0 \) and \( \lim_{x \to -\infty} f(x) = 0 \) since \( erf(0) = 0 \) and \( \lim_{x \to -\infty} erf(x) = 1 \). The first derivative of \( f(x) \) is,

\[
f'(x) = 2\left(\frac{1}{\sqrt{\pi}} - x\right)e^{-x^2}
\]

Therefore, (1). \( f(0) = 0 \) and \( f'(x) > 0 \) for \( 0 \leq x < \frac{1}{\sqrt{\pi}} \) indicates that \( f(x) \geq 0 \) when \( 0 \leq x < \frac{1}{\sqrt{\pi}} \); (2). \( f(1/\sqrt{\pi}) > 0, f'(x) < 0 \) for \( x > \frac{1}{\sqrt{\pi}} \) and \( \lim_{x \to -\infty} f(x) = 0 \) implies that \( f(x) \geq 0 \) when \( x \geq \frac{1}{\sqrt{\pi}} \). Taken together, \( f(x) \geq 0 \) i.e. \( 1 - erf(x) \leq e^{-x^2} \).

**Theorem 2.** The mean absolute error between the random diffusion distance \( L \) and the \( L_j, j = 1 \) to \( n \), induced by discretizing the probability density function \( p(L, \Delta t) = \frac{1}{\sqrt{\pi D \Delta t}} \exp(-L^2/4D\Delta t) \) into \( n \) bins of equal area and taking \( L_j \) as the midpoints of
the bins (in terms of area), is, smaller than or equal to \( \frac{\sqrt{\Delta t}}{n^2} \left( \sqrt{\ln(2n) + \frac{\sqrt{2}}{2}} \right) \) or \( \frac{L_4}{2n^2} \left( \sqrt{\pi \ln(2n)} + \frac{\pi}{2} \right) \).

Proof: As shown in equation (4.5), the probability function for \( L \) id essentially error function \( \text{erf}(\frac{L}{\sqrt{4D\Delta t}}) \). Hence, the mean absolute error between \( \frac{L}{\sqrt{4D\Delta t}} \) and its discretized counterpart \( \frac{L_j}{\sqrt{4D\Delta t}} \) is

\[
E \left| \frac{L}{\sqrt{4D\Delta t}} - \frac{L_j}{\sqrt{4D\Delta t}} \right| = \frac{1}{n} \sum_{j=1}^{n} \int_{\frac{j-0.5}{n}}^{\frac{j+0.5}{n}} \left| \text{erf}^{-1}(x) - \text{erf}^{-1}(\frac{j-0.5}{n}) \right| dx
\]

\[
= \frac{1}{n} \sum_{j=1}^{n-1} \int_{\frac{j+0.5}{n}}^{\frac{j-0.5}{n}} \left( \text{erf}^{-1}(\frac{j-0.5}{n}) - \text{erf}^{-1}(x) \right) dx
\]

\[
+ \frac{1}{n} \sum_{j=1}^{n} \int_{\frac{j-0.5}{n}}^{\frac{j+0.5}{n}} \left( \text{erf}^{-1}(x) - \text{erf}^{-1}(\frac{j-0.5}{n}) \right) dx
\]

\[
+ \frac{1}{n} \int_{\frac{n-0.5}{n}}^{\frac{n+0.5}{n}} \left( \text{erf}^{-1}(\frac{n-0.5}{n}) - \text{erf}^{-1}(x) \right) dx
\]

\[
+ \frac{1}{n} \int_{\frac{n-0.5}{n}}^{\frac{n+1}{n}} \left( \text{erf}^{-1}(x) - \text{erf}^{-1}(\frac{n-0.5}{n}) \right) dx
\]

\[
\leq \frac{1}{n} \sum_{j=1}^{n-1} \frac{0.5}{n} \left( \text{erf}^{-1}(\frac{j+0.5}{n}) - \text{erf}^{-1}(\frac{j-1}{n}) \right)
\]

\[
+ \frac{1}{n} \sum_{j=1}^{n-1} \frac{0.5}{n} \left( \text{erf}^{-1}(\frac{j}{n}) - \text{erf}^{-1}(\frac{j-0.5}{n}) \right)
\]

\[
+ \frac{0.5}{n^2} \left( \text{erf}^{-1}(\frac{n-0.5}{n}) - \text{erf}^{-1}(\frac{n-1}{n}) \right)
\]

\[
+ \frac{1}{n} \int_{\frac{n-0.5}{n}}^{\frac{n+1}{n}} \left( \text{erf}^{-1}(x) - \text{erf}^{-1}(\frac{n-0.5}{n}) \right) dx
\]

\[
= \frac{1}{2n^2} \text{erf}^{-1}(1 - \frac{0.5}{n}) + \frac{1}{n} \int_{\text{erf}^{-1}(1 - \frac{0.5}{n})}^{\infty} (1 - \text{erf}(x)) dx
\]

We now need to estimate \( \text{erf}^{-1}(1 - \frac{0.5}{n}) \) and \( f_{\text{erf}^{-1}(1 - \frac{0.5}{n})}^{\infty} (1 - \text{erf}(x)) dx \), separately.

Define \( g(x) = 1 - e^{-x^2} \). According to the Lemma 1, we have \( \text{erf}(x) \geq g(x) \). Since both \( \text{erf}(x) \) and \( g(x) \) are monotonically increasing functions, then

\[
\text{erf}^{-1}(y) \leq g^{-1}(y) = \sqrt{-\ln(1 - y)}
\]
Hence,

\[ erf^{-1}(1 - \frac{0.5}{n}) \leq \sqrt{-\ln \frac{0.5}{n}} = \sqrt{\ln(2n)} \]  

(4.8)

Using Lemma 1 again,

\[
\int_{erf^{-1}(1 - \frac{0.5}{n})}^{\infty} (1 - erf(x)) \, dx \leq \int_{erf^{-1}(1 - \frac{0.5}{n})}^{\infty} e^{-x^2} \, dx \\
= \frac{\sqrt{\pi}}{2} \left( \frac{2}{\sqrt{\pi}} \int_{0}^{\infty} e^{-x^2} \, dx - \frac{2}{\sqrt{\pi}} \int_{0}^{erf^{-1}(1 - \frac{0.5}{n})} e^{-x^2} \, dx \right) \\
= \frac{\sqrt{\pi}}{2} \left( 1 - erf(\frac{1}{\sqrt{n}}) \right) = \frac{\sqrt{\pi}}{4n} \]  

(4.9)

Therefore, by combining (4.8) and (4.9) the mean absolute error between \( L \) and \( L_j \) is estimated as:

\[ E|L - L_j| \leq \frac{\sqrt{D\Delta t}}{n^2} \left( \sqrt{\ln(2n)} + \frac{\sqrt{\pi}}{2} \right) \\
= \frac{L_d}{2n^2} \left( \sqrt{\ln(2n)} + \frac{\pi}{2} \right) \]  

(4.10)

This proves the theorem.  

For example, when we take \( n = 100 \) (i.e. area under the density function is partitioned into 100 bins of equal area), then the mean absolute error induced by the discretization process is smaller or equal to \( 0.032% \sqrt{D\Delta t} = 0.028%L_d \). In other words, the mean absolute error will be less than 0.03\% of the average diffusion distance \( L_d \).

In the Monte Carlo simulation of diffusion in three dimensional spaces, each Cartesian coordinate \((x,y,z)\) is, separately, either increased or decreased (randomly) by a distance \( L_j \).
Simulation of Receptor Binding

**Deterministic differential equation approach**

As a base model of a molecule (or agonist $A$) binding to its receptor, consider a case in which a monovalent agonist binds reversibly to a monovalent receptor $R$ to form a receptor/agonist complex $C$, with no further processes modifying this interaction:

$$ R + A \overset{k_f}{\underset{k_r}{\rightleftharpoons}} C $$

(4.11)

The association rate constant $k_f$ (Mol$^{-1}$ sec$^{-1}$) characterizes the velocity of the second-order interaction between the receptor and agonist, while the dissociation rate constant $k_r$ (sec$^{-1}$) characterizes the velocity of the first order breakdown of the receptor/agonist complex. According to the principles of mass action kinetics, the equation describing the time rate of change of the receptor/agonist complex density $C$ as a function of free receptor number $R$ and the agonist concentration $A$ is:

$$ \frac{dC}{dt} = k_f RA - k_r C. $$

(4.12)

Usually, the total number of receptors $R_T = R + C$ will not change during the course of binding/unbinding. If we also assume that the total amount of agonist (bound and unbound) $A_0 = A + C/N_A$ ($N_A$ is the Avogadro's number) is unchanged, then the equation can be easily solved:

$$ C(t) = C_0 \exp(-t/\tau) + \left( \frac{R_TA_0}{K_D + A_0} \right) \left[ 1 - \exp(-t/\tau) \right] $$

(4.13)

where $\tau = 1/(k_f A_0 + k_r)$, $K_D = k_r/k_f$ and $C_0$ is the initial complex density. The number of receptor-agonist complexes at equilibrium, $C_{eq}$, is in this case identical to
the steady-state value at which \( \frac{dC}{dt} = 0 \) and is approached for \( t \gg \tau \):

\[
C_{eq} = \frac{R_T A_0}{K_D + A_0} \tag{4.14}
\]

\( K_D \) is referred to as the *equilibrium dissociation constant*. It is frequently used as the parameter of binding affinity. As illustrated from the formula (4.14), 50% of the receptors will be agonist-bound at equilibrium if the initial concentration of agonists is \( K_D \).

**Probabilistic differential equation approach**

The probabilistic differential equation approach considers the association and dissociation events that occur during a short time interval \( \Delta t \). Instead of looking at the rate of change of the number of receptor/agonist complexes directly, this approach first defines the probability that there are \( C \) complexes at time \( t \), \( P_C(t) \). A kinetic equation can then be written, describing the changes in the number of complexes during \( \Delta t \):

\[
P_C(t + \Delta t) - P_C(t) = k_f A [R_T - (C - 1)] P_{C-1}(t) \Delta t - k_f A [R_T - C] P_C(t) \Delta t \\
- k_r C P_C \Delta t + k_r (C + 1) P_{C+1}(t) \Delta t + o(\Delta t)
\]

where \( o(\Delta t) \) represent terms of higher order in \( \Delta t \). In this equation, the first and second terms on the right-hand side represent the probability that there were \( C - 1 \) and \( C \) complexes, respectively, present at time \( t \) and one binding event occurred during \( \Delta t \); the third and fourth terms represent the probability that there are \( C \) and \( C + 1 \) complexes, respectively, present at time \( t \) and one dissociation event occurred. The limit of the difference equation as \( \Delta t \) approaches 0 yields differential equations:

\[
\frac{dP_C}{dt} = k_f A [R_T - (C - 1)] P_{C-1}(t) - k_f A [R_T - C] P_C(t)
\]
where $C = 1, 2, ..., (R_T - 1)$. The equations for $C = 0$ and $C = R_T$ are slightly different:

\[
\frac{dP_0}{dt} = -k_f A R_T P_0 + k_f P_1 \tag{4.16}
\]

\[
\frac{dP_{R_T}}{dt} = k_f A P_{R_T-1} + k_r R_T P_{R_T} \tag{4.17}
\]

This set of equations makes up the master equation. When the agonist concentration $A$ remains constant, they form a system of $(R_T + 1)$ coupled linear ordinary differential equations (ODE), which can be solved analytically if initial conditions are given. A possible set of initial conditions, describing the situation when no receptors are bound at time $t = 0$, is $P_C(0) = 0$ for $C \neq 0$ and $P_C(0) = 1$ for $C = 0$.

DeLisi and Marchetti (1983) provided solutions to these equations in their analysis of receptor binding fluctuations in chemotactic responses. We would like to discuss one of the ways to solve the system, i.e. using generation function method. The generating function, $G(s, t)$, is defined by following equation:

\[
G(s, t) = \sum_{C=0}^{R_T} s^C P_C(t)
\]

where $s$ is a dummy variable (Bharuca-Reid, 1960). The above set of linear ODEs can then be transformed into:

\[
\frac{\partial G}{\partial t} = (1 - s) \left[ (k_f A s + k_r) \frac{\partial G}{\partial s} - (k_f A R_T) G \right]
\]

The initial condition for $G(s, t)$ can be obtained from that for $P_C(t)$:

\[
G(s, 0) = \sum_{C=0}^{R_T} s^C P_C(0) = 1
\]
The boundary condition, on the other hand, is found by the requirement that all probabilities must sum to one:

\[ G(1, t) = \sum_{C=0}^{R_T} P_C(t) = 1 \]

The solution \( G(s, t) \), once found, can then be used to recover the individual probabilities by the formulae:

\[
P_0(t) = G(0, t) \]
\[
P_C(t) = \frac{1}{C!} \left[ \frac{\partial^CG}{\partial s^C} \right]_{s=0}
\]

Two quantities of more interest are the expected (mean) value of \( C \), denoted \( \bar{C} \), and the variance \( \sigma_C^2 \). They are defined and related to generating function by:

\[
\bar{C} = \sum_{C=0}^{R_T} CP_C = \left[ \frac{\partial G}{\partial s} \right]_{s=1}
\]
\[
\sigma_C^2 = \sum_{C=0}^{R_T} (C - \bar{C})^2 P_C = \left[ \frac{\partial^2 G}{\partial s^2} + \frac{\partial G}{\partial s} - \left( \frac{\partial^2 G}{\partial s^2} \right)^2 \right]_{s=1}
\]

Using the approach given above, we can find the solutions:

\[
\bar{C}(t) = \frac{R_TA}{K_D + A} \left[ 1 - \exp\left( -t/\tau \right) \right]
\]
\[
\sigma_C^2 = \frac{R_TA}{(K_D + A)^2} \left[ A \exp\left( -t/\tau \right) + K_D \left[ 1 - \exp\left( -t/\tau \right) \right] \right]
\]

Note that the solution is the same as the solution to the deterministic binding model with no initial number of complexes \( (C_0 = 0) \). Here, we are more interested in the steady state solution, the same as equilibrium in this case, arising from setting \( \partial G/\partial t = 0 \) or taking the limit of the transient solution as \( t \) approaches infinity:

\[
\bar{C}_{eq} = \frac{R_TA}{K_D + A}
\]
\[
(\sigma_C^2)_{eq} = \frac{R_TAK_D}{(K_D + A)^2}
\]
where $\tau = k_f A + k_r$. The interesting and important observation is that the statistical variance expected at equilibrium binding is proportional to the total number of cell receptors, $R_T$. The root-mean-square deviation, $\delta C_{eq} = (\sigma_{C_{eq}})$, is equal to $(R_T A K_D)^{1/2}/(K_D + A)$. A useful expression, describing the expected relative root mean square fluctuation in equilibrium complex number due to stochastic effects in binding, can then be written:

$$\frac{\delta C_{eq}}{C_{eq}} = \left( \frac{K_D}{AR_T} \right)^{1/2}$$

(4.22)

**Monte Carlo simulation of receptor binding**

Instead of formulating the “population probability” (i.e. the probability that the number of complexes formed at time $t$ is $C$), the Monte Carlo method focuses on the probability that an individual binding reaction will occur within a time step $\Delta t$. It is assumed that the average probability, to the first order in $\Delta t$, that a binding reaction will occur in the next time interval $(t, t + \Delta t)$ is $p_b(\Delta t) \equiv k_f A \Delta t$, where $A$ is the agonist concentration in the “vicinity” of the receptor. To formulate the exact expression for $p_b(\Delta t)$, we calculate $p_{nb}(\Delta t)$, the probability that no binding reaction occurs in $\Delta t$. Imagine the interval $(t, t + \Delta t)$ to be divided into $M$ subintervals of equal length $\epsilon = \Delta t/M$, the probability that no reaction occurs in $\epsilon$ should be $1 - k_f A \epsilon + o(\epsilon)$. Since there are $M$ such subintervals between $t$ and $t + \Delta t$, then

$$p_{nb} = [1 - k_f A \epsilon + o(\epsilon)]^M$$

This is true for any $M > 1$, and in particular it is true in the limit of infinity large $M$. Hence,

$$p_{nb} = \lim_{M \to \infty} [1 - (k_f A \Delta t + o(M^{-2}))/M]^M$$
Therefore,

\[ p_b = 1 - e^{-k_f A\Delta t} \]  \hspace{1cm} (4.23)

In Monte Carlo simulation of binding, a uniformly distributed number \( r \) will
be generated from \([0,1]\) and compared with the \( p_b \). If \( r \) is smaller than \( p_b \), binding
occurs in \((t, t\Delta t)\). If, on the other hand, \( r \) is larger that \( p_b \), then there will be no
binding allowed in the time step. The theoretical background behind the simulation
procedure can be proved as follows. Before we present the proof, let us first look at
the more general situation (Gillespie, 1976). The inversion method for generating a
random discrete number \( i \) according to probability density function \( P(i) \) is to draw
a random number \( r \) from the uniform distribution in the unit interval and take for \( i \)
that value which satisfies

\[ F(i - 1) < r \leq F(i) \]

The proof of this general scheme was given by Gillespie (1976) and can be found in
Appendix A. Now, we will apply the scheme to the simulation of binding.

**Theorem 3.** To simulate the binding probability \( p_b \) is to draw a random uniformly
distributed number \( r \) from \([0,1]\) and allow binding if the \( r \) is smaller than \( p_b \) and no
binding otherwise.

**Proof:** To simulate the binding reaction using the Monte Carlo method is essentially
to generate a binomial random variable \( i \) with following density function:

\[ P(i = 0) = p_b; \quad P(i = 1) = 1 - p_b \]
where \( P(i = 0) \) and \( P(i = 1) \) are the probabilities that binding will and will not occur within \( \Delta t \), respectively. The distribution function for the binomial variable is:

\[
F(i = 0) = p_b; \quad F(i = 1) = 1
\]

According to the general scheme given above, we need to choose the \( i \), which satisfies \( F(i - 1) < r \leq F(i) \) after substituting \( r \) generated from \([0,1]\). It is obvious that only when \( i = 1 \) can we draw such \( r \) that both of the inequalities are satisfied. That is, we do not allow binding \((i = 1)\) when \( p_b < r \leq 1 \). In other words, binding should be allowed at the start of the \( \Delta t \) when \( r < p_b \leq 1 \). #

Monte Carlo Simulation of GABA\(_A\) Synapse

**Kinetic model of GABA\(_A\) receptors**

Single channel recording revealed bursts of openings of GABA\(_A\) receptor-ion channel complexes in the presence of GABA. It is believed that the receptor has two GABA-binding sites. Various kinetic models of the GABA\(_A\) receptor have been proposed to fit the experimental data from single channel recordings of different preparations. It may be noted that more than one kinetic model can be used to fit the same single channel recording results. The discussion of exactly how the kinetic models are obtained from the experimental data and their uniqueness is out of the scope of the study. We will focus on the GABA\(_A\) receptor kinetic model of cultured mouse spinal cord neuron (Fig.4.1, Twyman et al., 1992). The kinetic model has a total of 14 states, which are divided into three categories: open states, closed states, and a desensitized state. The receptor has three open states, of which one is single-
bound and two others are double-bound. Closed states have also been subdivided into extraburst closed states and intraburst closed states. The desensitized state has been proposed to explain the observation that GABA-induced whole-cell current decays rapidly in the prolonged presence of GABA. The complexity of such a model is one more factor to discourage the attempt to analytically solve the receptor kinetics using the deterministic differential equation approach.

Monte Carlo simulation of GABA diffusion and binding

Based on the published data regarding the morphology of GABAergic synaptic buttons and at the same time taking simulation simplicity into consideration, a model synapse is assumed to be bounded by two square pre- and postsynaptic membranes of 400 × 400 nm each, with a gap width of 20 nm.

In our Monte Carlo simulation of GABAergic synaptic transmission, GABA molecules in one vesicle or more are released at a point source from the presynaptic membrane. GABA_A receptors (GABAR) on the postsynaptic membrane are modeled as small squares (assumed to be the same size as Ach receptor, i.e. 8.5 × 8.5 nm), each of which is enclosed in a bigger square of side length $S_{qr}$. The side length $S_{qr}$ can be found from $1/S_{qr}^2 = \sigma_r$, where $\sigma_r$ is the density of GABA_A receptors. There is no data at the present time specifying the receptor density $\sigma_r$ at the GABA_A synapse. However, it has been postulated that, at least in some synapses, GABA_A receptors might distribute on postsynaptic membrane in clusters (or “bunches”) (Otis et al., 1994). Hence, in the Monte Carlo simulation studies, $S_{qr}$ is taken as 10 nm to leave a small space between receptors.

After each time step $\Delta t$, when a GABA molecule has moved a distance $L_j$ (as
Figure 4.1: Kinetic model of \( \text{GABA}_A \) receptor
determined by the scheme described above), an imaginary line joining the old and new position of the GABA molecule is examined. If the molecule has hit or crossed any bounding surface where there is no GABAR or if the molecule has hit a GABAR which is already fully bound with two GABA molecules then specular reflection is specified. That is, in the two dimensions parallel to the postsynaptic membrane the molecule is allowed to move in the same way as before, but in the dimension perpendicular to the membrane it is only allowed to move in the reverse direction after the hit or imaginary cross. In other words, the molecule will bounce off the surface of the postsynaptic membrane in the next time step. If, on the other hand, the GABA molecule had hit a GABAR that has at least one free binding site, an uniformly distributed random number $r$ will be chosen between 0 and 1. If $r$ is less than $p_+$, a new Monte Carlo binding probability per time step $\Delta t$ as formulated below, then the GABA molecule is allowed to bind; if the random number is between $p_+$ and 1, the GABA is again specified as bouncing off the surface.

To calculate the Monte Carlo binding probability $p_b = 1 - e^{-k/A\Delta t}$ derived above, one has to find the GABA concentration $A$ in the "vicinity" of the GABAR. For the following two reasons we prefer a new Monte Carlo binding probability, $p_+$, which does not involve the agonist concentration $A$: (1) the whole purpose of simulating binding using the Monte Carlo method is to focus on individual molecules, receptors and their binding reactions, and to evaluate their roles in shaping the IPSCs; (2) it is not an easy task to accurately track the GABA concentration in the "vicinity" of each receptor during simulation runs since it is really difficult to decide the proper boundaries of a receptor "vicinity". Before we start to formulate the new Monte Carlo binding probability $p_+$, it is very helpful to look at $p_b$ from a different angle.
The probability \( p_b = 1 - e^{-k_f A \Delta t} \) is essentially the number of bindings that can occur in one time step.

To formulate the new binding probability, let us consider the subpopulation of GABA molecules within the small cubic volume sitting above one receptor square and having a height \( L_d \) (i.e., with volume \( S_{qr}^2 \times L_d \)), the average diffusion length in one time step. Assume there is no interactions between molecules in diffusion, then half of all the GABA molecules within this small volume will, on average, hit during one time step \( \Delta t \) the receptor square. Thus the number of GABA molecules that hit the GABAR square per \( \Delta t \) is \( 0.5N_a A \) (where \( A \) is the molar concentration of GABA within that volume and \( N_A \) is the Avogadro’s constant) times \( S_{qr}^2 L_d \) (i.e., the volume above the GABAR square). The product of this number of hits and the probability of binding, \( p_+ \), gives the number of bindings per time step. Now, chemical kinetics requires that the number of bindings per GABAR square in \( \Delta t \) be equal \( 1 - e^{k_f A \Delta t} \), which is approximated as \( k_f A \Delta t \) for “small enough” \( \Delta t \) (the error produced by this approximation is estimated in a theorem given below and is used as one of the factors to be considered in choosing \( \Delta t \)). Therefore, the number of bindings per time step must equal \( k_f A \Delta t \):

\[
0.5N_a AL_d S_{qr}^2 p_+ \approx k_f A \Delta t
\]  

(4.24)

Using \( S_{qr}^2 = 1/\sigma_r \), we then get two alternative forms of binding probability:

\[
p_+ \approx \frac{k_f \sigma_r (\pi \Delta t)^{\frac{1}{2}}}{N_a D_\frac{1}{2}}
\]  

(4.25)

\[
p_+ \approx p_+(L_d/S_{qr})
\]  

(4.26)

where \( p_{+0} \) is the Monte Carlo binding probability for the special average step length \( L_d = S_{qr} \), given by \( p_{+0} = (k_f/N_a)(\pi/2DS_{qr}) \).
Theorem 4. The first order approximation of \( p_b = 1 - e^{-k_f A\Delta t} \) as \( k_f A\Delta t \) produces in \( p_+ \) an absolute error of order \( \Delta t^{\frac{3}{2}} \).

Proof: Without the first order approximation to \( p_b \), the accurate equation related to (3.24) is:

\[
0.5N_aAL_dS^2_{q,r}p'_+ = 1 - e^{-k_f A\Delta t}
\]

where \( p'_+ \) is the “accurate” expression for \( p_+ \). Then,

\[
p'_+ = \frac{k_f\sigma_r(\pi\Delta t)^{\frac{1}{2}}}{N_aD^{\frac{1}{2}}} + \frac{2\sigma_r}{N_aAL_d} \sum_{i=2}^{\infty} \frac{(-k_f A\Delta t)^i}{i!}
\]

Hence, the absolute error in \( p_+ \) is

\[
|p_+ - p'_+| = \left| \frac{2\sigma_r}{N_aAL_d} \sum_{i=2}^{\infty} \frac{(-k_f A\Delta t)^i}{i!} \right|
\]

\[
= \left| \frac{\sigma_r(k_f A\Delta t)^2}{N_aAL_d} + o(\Delta t^3) \right|
\]

\[
= \left| \frac{\pi^{\frac{1}{2}}\sigma_r k_f^2 A\Delta t^{\frac{3}{2}}}{2N_aD^{\frac{1}{2}}} + o(\Delta t^{\frac{3}{2}}) \right|
\]

This proves that the absolute error in \( p'_+ \) is of order \( \Delta t^{\frac{3}{2}} \). 

Destinations of the GABA molecules in synaptic juction

The fate of neurotransmitters in the synaptic junction following their release is very important in determining the time course of postsynaptic responses. Unlike the Ach synapse, where degradation of Ach molecules by Ach esterases is the major factor contributing to the disposal of the transmitter, at GABA_A synapse there are two major pathways, re-uptake by the presynaptic membrane and diffusing out of the synaptic junction. Of the two factors, experimental data supports the idea that
the diffusion of GABA molecules out of the synaptic junction happens so fast that it plays a more significant role in shaping the time course of IPSC, compared with the GABA re-uptake (Mody, et al., 1994). Furthermore, it has been reported that the inhibitors of the re-uptake process failed to modify the GABAergic synaptic transmission in (Otis and Mody, 1992; Thompson and Gahwiler, 1992). For these reasons, the re-uptake of GABA will not be considered in our simulation study. Instead, diffusion-out-of-the-synaptic-gap will be the only process contributing to the “disposal” of GABA molecules. More specifically, a GABA molecule will be taken out of the simulation if it diffuses out the synaptic gap.

Monte Carlo simulation of receptor unbinding and state transitions

If a molecule is bound to a receptor, its chemical kinetic rate constant for unbinding, $k_r$, is converted into a Monte Carlo probability for unbinding $p_-$. Per time step, $p_- = 1 - \exp(-k_r \Delta t)$, which is close to $k_r \Delta t$ if $\Delta t$ is small compared with 1. Similarly, to determine whether a molecule unbinds during a time step we again randomly choose a number between 0 and 1, and allow unbinding if it is between 0 and $p_-$ and retain binding if it is between $p_-$ and 1. Upon unbinding, a GABA molecule is moved by an average step length ($L_d$) from GABAR to which it had been bound. In actual simulation, a distinction has to be made between single-bound and double-bound GABAR with a corresponding suffix 1 or 2 for $p_+/p_-$ and $k_f/k_r$. The transition between different states of the receptor will be simulated in a similar fashion as the receptor unbinding, i.e., the transition kinetic rate constant $k_t$ will be converted into Monte Carlo probability of transition, $p_t = 1 - \exp(-k_t \Delta t)$. Note that the agonist concentration $A$ is absent in both probability expressions.
Choosing $\Delta t$

The computational cost for a IPSP simulation is inversely proportional to the time step $\Delta t$. It would be obviously too costly to mimic the actual Brownian motion of molecules, which is in the range of $10^{-9}$ sec (Bartol et al., 1991). Moreover, as shown below, it is really not necessary to use a $\Delta t$ this small in simulation.

First of all, $\Delta t$ and $L_d$ have to be chosen small enough so that all the probabilities (including binding/unbinding and state transition) are smaller than one. For typical values of $k_f$ for GABA to GABAR binding ($2 \times 10^7$ Mol$^{-1}$sec$^{-1}$ in the kinetic model shown in Fig.4.1), $p_{+0} \ll 1$ so that $L_d$ can be chosen several times as large as $S_{qr}$ (=10 nm in our simulations) and still have $p_+ < 1$. For example, the choices of $\Delta t = 0.1$ and $1 \mu s$ make $L_d \approx 8.7$ and 26 nm, respectively. Such choices of $\Delta t$ result in all of the unbinding and state transition probabilities (calculated using the rate constants in Fig.3.1) far below one.

The rise time of IPSO, $t_r$, which is usually a few milliseconds, is a very important parameter subject to physiological and pharmacological modulations. Obviously, $\Delta t$ has to be small compared with the time over which bulk conditions change appreciably: i.e., $\Delta t \ll t_r$. A choice of $\Delta t$ in microsecond range will easily satisfy this requirement.

The considerations for $\Delta t$ discussed above are more or less qualitative in nature. The mathematically strict limitations on choice of $\Delta t$ come from the error bounds (4.10 and 4.27). The mean absolute error induced by discretization of the diffusion distribution function, for a choice of $\Delta t = 0.1$-$1 \mu s$, is about 0.028$\%L_d$ or 0.0025-0.0076 nm. According to Theorem 4, the error induced by the approximation of binding probability $p_+^r$ as $p_+$ is $(\pi^{\frac{1}{2}} \sigma_r k_f^2 A \Delta t^2)/(2 N_a D^\frac{1}{2}) + o(\Delta t^\frac{1}{2})$. For typical values
of \( \sigma_r = 10^{12}/\text{cm}^2 \) and \( k_f \), the choice of \( \Delta t \) from to 0.1 to 1 \( \mu \text{s} \) results in an error of order \( 10^{-10} \) to \( 10^{-8} \) in \( p_+ \), if an average concentration of 1 mM GABA is assumed. Taken together, \( \Delta t \) in the range from 0.1 to 1 \( \mu \text{s} \) will be used in the following simulation studies.

Validation of Methods

Before we proceed to use the Monte Carlo method for the simulation of GABAergic synaptic transmission, we need to validate the method. Specifically, we need to test the validity of the simulation scheme in modeling of molecule diffusion and receptor binding/unbinding. The basic idea behind the tests is to compare the simulation results obtained using the Monte Carlo method with that using deterministic differential equation approach. A previous study has provided test results for diffusion and unbinding rate (Batto et al., 1991). We will, in this study, reproduce the diffusion test results and examine the binding/unbinding events for a population of receptors when they are exposed to a constant concentration of agonists, a condition commonly referred to as concentration clamp.

Diffusion test

Consider the diffusion of molecules in three dimensional open space with no reflecting walls. Suppose the molecules are released at the same point source in the space and were then allowed to diffuse for a certain number of time steps. Using the deterministic differential equation approach, the distribution of the molecules is known analytically in this spherical coordinate system (Crank, 1975). The volume density \( A(r,t) \) as a function of radial distance \( r \) from the point source at time \( t \),
Figure 4.2: Comparison of the simulation results of molecule diffusion in an open spherical coordinate system obtained using a deterministic differential equation (solid line) and a Monte Carlo method (circles). Shown in the graph are the distance distribution of 10000 molecules at 0.3 ms, released at a point source (origin) at time $t = 0$. The thickness of the "shells" (explained in the text) is 100 nm, $D = 6 \times 10^{-6} \text{cm}^2/\text{s}$, and $\Delta t = 0.1 \mu\text{s}$ which determines $L_\theta \approx 8.9 \text{ nm}$.

should be:

$$A(r, t) = N_0 (4\pi D)^{-3/2} \exp(-r^2/4Dt), \tag{4.28}$$

where $D$ is the diffusion constant, $N_0$ is the total number of molecules initially released and $r = (x^2 + y^2 + z^2)^{1/2}$. To test the validity of the Monte Carlo method for diffusion simulation, the sphere is separated into shells of equal thickness centered at the point source. Numerical runs are carried out to trace the locations of the molecules and compute the concentrations in each shell. The molecule concentrations are then compared with the analytic results obtained using the formula described above.
Results of the diffusion test are demonstrated in Fig. 4.2. At “larger” radial distances, a very good agreement was found between the concentration distributions (measured in number of molecules/μm²) simulated either with the Monte Carlo method or from the analytic solution (4.28). However, as shown in the Fig. 4.2, larger discrepancy exists for small distances r between the results obtained with the two methods. This error represents an intrinsic limitation of the Monte Carlo method due to the “Poisson fluctuation”, ±Ni^{1/2}/4π²Δr, where Ni is the number of molecules in ith shell, and ri and Δr are the inner radius and thickness of the shell, respectively. When Ni is small (i.e., close to the point source), the fluctuation in the number of molecules per volume is large.

**Binding/Unbinding test**

To test the validity of the Monte Carlo scheme in the simulation of binding/unbinding using Monte Carlo method, suppose a population of free GABA_A receptors (900 of them in this case) were exposed to a constant concentration of GABA molecules at time t = 0. Since in this test study we were only interested in the binding/unbinding events, we considered exclusively the first step in the GABA_A receptor kinetic model, i.e.,

\[ R + GABA \xrightarrow{k_f} GABA \cdot R \]

Using the deterministic differential equation approach, the number of agonist-receptor complexes as a function of time could be obtained analytically using formula (4.13). The analytic result was then used as a criterion to judge the validity of the Monte Carlo method in binding/unbinding reaction simulation.
Figure 4.3: Comparison of the first step binding/unbinding kinetics of GABA receptors modeled with deterministic differential equation (solid) and Monte Carlo Method (circles). Shown in the graph are the time courses for the number of agonist-receptor complexes formed when 900 GABA_A receptors are exposed to GABA of constant concentration (50μM). Δt = 0.1μs and K_D = k_r/k_f = 1.25μM
Presented in Fig.4.3 is the comparison of the time courses of agonist-receptor complexes simulated using either the Monte Carlo method or the analytic formula (4.13). Excellent agreement is evident at both the transient and steady phases of the solutions. It is worthwhile to note that the Monte Carlo simulation results illustrated in the figure are only from one computation run and no averaging among runs was done.

Simulation Results

The traditional way of investigating GABA_A receptor-mediated synaptic transmission was to study the IPSCs or IPSPs elicited by a stimulating electrode placed in the vicinity of the recording electrode. The responses elicited by stimulations are usually of high amplitude. Since the refinement of patch-clamp recordings in brain slices, small IPSCs resulted from spontaneous release of GABA from presynaptic terminals are commonly detected. These spontaneous events are referred to sIPSC. They persist at least partially even after the application of tetrodotoxin (TTX) to block the propagation of action potentials to the terminals. Therefore, the sIPSCs resistant to TTX are more commonly termed as miniature IPSCs or mIPSCs by analogy to the miniature currents recorded at the neuromuscular junction in the presence of TTX.

The following Monte Carlo simulation studies were designed to examine the role of the time course of GABA release, GABA concentration in the cleft, and GABA_A receptor population, kinetic states and distribution in shaping the mIPSCs. Experimental observations were often used as the criteria to predict the implication of the simulated responses. The complete kinetic model of GABA_A receptors in the cultured mouse spinal neurons shown in Fig.4.1 was utilized to examine the questions
Figure 4.4: Top: Synaptic response to 5000 GABAs simultaneously released at $t = 0$. A total of 400 GABA$_A$ receptors are assumed uniformly distributed on the postsynaptic membrane (refer to the text for other details). Bottom: Time courses of the number of open channels bound with one or two GABAs following the release of GABAs. $\Delta t = 0.1\mu s$. The data is sampled at 0.1 ms/point or 10 kHz. n=20

concerning GABAergic synaptic transmission.

**Synaptic response to simultaneously released 5000 GABAs**

In this subsection, GABA$_A$ receptor-mediated synaptic response to the release of GABA molecules in one vesicle was simulated in the model synapse. Since there is no report so far specifying the exact number of molecules in a GABA vesicle and it was suggested that there are approximately 5000 molecules in one small Ach vesicle (Bartol et al., 1992), an assumption of 5000 GABA molecules was made for one GABA vesicle in the simulation. We further assumed that there is a cluster of 400
GABA receptors distributed uniformly on the central square patch (200 x 200 nm) of postsynaptic membrane (one receptor on every square membrane patch of 10 x 10 nm in size). The Monte Carlo analysis as discussed above was used to trace the states of each GABA molecule and the states of receptors.

At time $t = 0$, 5000 GABAs are simultaneously released from the geometric center of the square presynaptic membrane, which produced the simulated response of the postsynaptic membrane (Fig.4.4, top). The peak conductance of the response is $G_{Cl^-} = 350.0 \mu S$, which corresponds to a chloride current of $I_{Cl^-} = G_{Cl^-} \times (V_m - V_{Cl^-}) = 7.0 \mu A$ if the driving force for the chloride ion is $(V_m - V_{Cl^-}) = 20 \text{ mV}$, where $V_m$ and $V_{Cl^-}$ are the resting membrane potential and chloride reversal potential of the postsynaptic cell, respectively. The initial rising rate (slope of response at $t=0$)
is about 300 pS/ms. Time courses of the open channels are shown at the bottom of Fig.4.4. As expected, double-bound open channels are the main population of channels contributing to the response. The features of single channel recording can be also found in the graph. The short-lived switches in the number of open channels, highlighted with a "*" in the graph, are presumably due to the transitions between open and intrabust closed states.

There is a great interest in the binding dynamics of receptors and the destination of GABAs molecules following their release since this information will help to predict the role of such factors as the probability of presynaptic release and the accumulation of transmitters in shaping postsynaptic responses. Shown in Fig.4.5 is the fate of single- or double-bound receptors (top) and the GABA molecules bound on a receptor or diffused out of synaptic gap (bottom). It is clear that "long" before the peak response is reached at 4.1 ms, over 90% of GABAs diffused out of the synaptic junction 40 µs after their release from presynaptic membrane. Furthermore, there are a maximum of 289 receptors either single- or double-bound at t=70 µs, representing the 72% of total receptors.

Another quantity of interest in the investigations of synaptic transmission is the efficacy of receptors, i.e. the number receptors in open states at the peak IPSCs, relative to the total available receptors. It has been suggested that there might be 60-80 receptors opening at the peak of the mIPSCs (Mody et al., 1994). In the simulation of the synaptic response to simultaneously released 5000 GABAs, at the peak response, 116 receptors are bound with one GABA molecule, of which 5 are in open states, and 26 receptors are bounded with two GABAs, of which 9 are in open states.
Effect of the increase in the number of available postsynaptic GABA<sub>A</sub> receptors

There are a number of factors affecting the potency of synaptic transmission at GABA<sub>A</sub> synapses. An increase in the number of GABA<sub>A</sub> receptors is believed to mediate the long-term modulation of inhibitory synaptic transmission in granule cells of the dentate gyrus during kindling-induced epilepsy (Otis et. al, 1994). The Monte Carlo simulation provides a convenient way to examine the consequences of a change in the number of available receptors.

Suppose that there are now 1600 GABA<sub>A</sub> receptors distributed uniformly, i.e., one receptor on every small patch of 10×10 nm on the postsynaptic membrane, and
all of the other conditions are the same as in Fig.4.4. Shown in Fig.4.6. (top) is the comparison of the responses of the 1600 and 400 GABARs to the central release of 5000 GABAs. For the 3-fold of increase in the number of free GABARs, the peak conductance response is increased by only about 60% to 559.65 pS when 11 single-bound and 12 double-bound receptors are open. The initial rate of rising in the response is about 498 pS/ms, a similar 60% increase.

Considering the biological cost in synthesizing the extra 1200 GABARs, increasing the number of receptors does not seem to be a very efficient way to enhance the simulated synaptic responses. The main reason behind the “inefficiency” of the increase in the number of available receptors is due to the fast diffusion of a majority of GABAs out of synaptic gap following their release (Fig.4.5 and Fig.4.7, bottoms).
The maximum number of bound receptors, in the simulation of response of the 1600 GABARs to a simultaneous release of 5000 GABAs, is 584, far below the 80% receptor saturation level predicted by experimental studies (Colquhoun and Sakmann, 1985). The lack of receptor saturation in the simulation of synaptic response to 5000 GABAs further supports the idea that there might be more than one vesicle of GABAs released in producing mIPSCs recorded experimentally. For these reasons, we will examine the consequences of simultaneous or consecutive release of vesicles.

Response to simultaneous release of 50000 GABAs

The response of 400 GABARs to a simultaneous release of 50000 GABAs into the model synapse is simulated under the same conditions as stated in Fig. 4.4. The peak response (1016.35 pS) is reached at 3.1 ms, which represents about a 3-fold increase in comparison with the peak response of the 400 receptors to 5000 GABAs (Fig. 4.8, top). Of the receptors in open state, the double-bound receptors contribute in a significantly greater proportion to the response (Fig. 4.8, bottom). At the peak, while there are 33 double-bound receptors in open state, only 6 single-bound GABARs are open. The overall number of double-bound receptors is also greater than that in single-bound state especially in the first 1-2 ms following the release (Fig. 4.9, top).

Although over 90% of GABAs diffused out of the synaptic junction about 50 μs after the release of the transmitters (Fig. 4.9 bottom), the overall number of bound receptors (single or double) in the initial 2 ms is well above the 80% saturation level predicted by experimental studies (Fig. 4.9, top). However, the number of receptors in open states (39) at the peak response is still far below the experimentally predicted 60-80 open receptors. These results suggest that the “simultaneous release” (in math-
Figure 4.8: Comparison of the simulated responses of 400 GABARs to 5000 (dashed) or 50000 (solid) centrally released GABAs at t=0 (top), and time courses of corresponding single(solid)- or double(dashed)-bound GABA\textsubscript{A} open channels (top) for the latter case. n=20
Figure 4.9: Time courses of single(solid)- or double(dashed)-bound and total bound (dots) GABA<sub>A</sub> receptors (top) and GABA molecules bound (solid) or diffused out (dashed) (bottom), following the release of 50000 GABAs at t = 0, n=20.

Mathematical sense) of 50000 GABAs (about the amount in 10 vesicles) seems unlikely to happen at a living synapse. It is reasonable to think that the time interval(s) between the release of different pools (vesicles or cluster of vesicles) of GABA could easily exceed tens of microseconds. The consecutive release of GABA pools will keep the transmitter concentration in the synapse consistently high at least for a short period of time. It has been suggested that during mIPSCs, the concentration of GABA in the synaptic cleft might be in the millimolar range (Maconochie, et. al. 1994). In the next section, the effect of a short presence of consistently high concentration of GABAs in the cleft on synaptic responses was simulated. Note that for the volume in the model synaptic gap, 1 mM concentration corresponds to 1927 GABAs.
Figure 4.10: Top: Superimposed responses of 400 GABARs to 1 ms GABA (1 mM) clamp (solid, explained in the text) or release and free diffusion of 5000 GABA (dashed); Bottom: Time courses of single(solid)- or double(dashed)-bound GABA\textsubscript{A} open receptors in the case of GABA clamp.

**Receptor saturation and GABA efficacy at GABA\textsubscript{A} synapse**

To simulate the effect of a short clamp of high concentration of GABA (in mM range) on the synaptic response, a simple scheme is used: 1927 GABAs (i.e. 1 mM) are released simultaneously at $t=0$ from the center of presynaptic membrane, and allowed to diffuse (but not out of the junction), bind and activate the GABARs for 1 ms. Binding of one GABA to a receptor will prompt the adding of another GABA into the pool of free GABAs in the next time step $\Delta t$ in order to ensure that the overall concentration of free GABA in the cleft is clamped at 1 mM. In contrast, unbinding of a GABA molecule from a receptor will result in removal of that GABA from the synaptic junction. At the end of the 1 ms, all of the free GABAs still left in
the junction or the GABAs dissociating from the receptors later are allowed to diffuse “freely”, i.e., if any GABA diffusing out of the synaptic gap will be taken out of simulation in the next time step.

The simulated response of 400 GABARs to the 1 mM GABA (1927 molecules) clamp for 1 ms is compared (Fig.4.10) with that to the release and “unrestricted” diffusion of 5000 GABAs simulated in Fig.4.4. The short persistent presence of high concentration of GABAs produced a response that is nearly 120% larger at the peak (767.95 pS at 3.5 ms). At the peak response, there are 178 single-bound receptors, of which 6 are open, and 93 double-bound receptors, of which 24 are open (Time courses of the single-bound and double-bound receptors are demonstrated in Fig.4.11). Keeping in mind the fact that the number of molecules in 1 mM GABA is less than the 5000 GABAs, the GABA efficacy is hence increased by a percentage greater than 120%. At 1.2 ms, there are a maximum of 369 bound receptors (single or double) (Fig.4.11), which as predicted is below the 98% receptor saturation level observed at Ach synapse. However, as stated above, the number of open channels (30) at the peak response is still “far” blow the predicted efficacy (60-80). These results suggest that during mIPSC either that the concentration of GABA persistently present for a short time is more than 1 mM or the duration of the high GABA is longer that 1 ms or that the number of receptors exceeds 400. The next computation runs were designed to simulate the response of 1600 receptors (uniformly distributed as described in subsection 2) to the short (1 ms) presence of high (1 mM) concentration of GABA.

Shown in Fig.4.12 is an illustration of the simulation. The peak response of the 1600 GABARs (2862.75 pS) is reached at 3.4 ms when 26 single- and 87 double-bound
Figure 4.11: Fate of single(solid)- or double(dashed)-bound GABA_A receptors (top) and GABA molecules bound (solid) or diffused out (dashed) (bottom), when 1927 GABAs are induced into the model synaptic junction at t=0 and not allowed to diffuse out for 1 ms. n=20.
Figure 4.12: Top: Superimposed responses of 1600 (solid) or 400 (dashed) GABARs to 1 ms GABA clamp (1 mM); Bottom: Time courses of the single(solid)- or double(dashed)-bound open channels in the case of 1600 GABARs. n=20
receptors are open. The peak response represents a nearly 300% increase compared with that of the 400 GABA receptors. There are a maximum of 1468 bound receptors at 1.1 ms, corresponding to about 90% of saturation level. These results demonstrate that if the GABA concentration in the synaptic gap stays consistently high even for a short period of time (1ms) the ability of cells to increase the GABAergic synaptic transmission through increasing the number of available receptors is greatly enhanced. Specifically, a nearly 300% of increase in the peak amplitude is expected for a 3 fold of increase in GABA_A receptors.

The 113 open receptors at the peak conductance are well above the range of experimentally predicted number of activated receptors. This suggests that if the 1 mM GABA clamp for 1 ms closely reflects the condition in the synaptic junction during mIPSCs the number of available receptors at GABA_A synapse should be less than 1600. To test this idea, the response of 900 receptors to the 1 ms clamp of 1 mM GABA was computed. At the peak response (1604.40pS at 4.2 ms), there are 16 single- and 47 double-bound open channels, which matches well with the predicted value of 60-80 (not shown).

Role of desensitized and intraburst closed states in producing IPSCs

In the Monte Carlo simulation of GABA_A receptor-mediated IPSCs, the role of a kinetic state in producing IPSPs can be easily studied by simply comparing the simulated IPSCs before and after removing the state from the kinetic scheme. This way of examining the role of states may not be feasible for some states which lie in the core of the kinetic scheme (for example, all the open states). The desensitized and intraburst closed states, however, are related to only one other state. Therefore,
Figure 4.13: Comparison of the simulated responses of 1600 GABA$_A$ receptors with (solid) or without (dots) the state D to the continuous presence of 100 μM of GABA, starting at $t = 0$. $\Delta t = 1\mu$s and data sampling rate is 1 ms/point.

the effects of these two sets of states on IPSCs can be tested.

a. Desesitized state

A variety of electrophysiological studies, including the experiments that I have conducted, utilize isolated cell preparations, in which all synaptic contacts have been dissociated. Since in this case there is no presynaptic source for neurotransmitter release, a pressure-ejection system is often used to deliver a flow of chemical (GABA in my case) to a single neuron (held at a constant voltage) so that the cell is exposed to the chemical of constant concentration, a condition termed concentration clamp.

The membrane current, recorded under the concentration-clamp (and voltage-clamp) condition, is mediated by all the receptors on the neuron, hence the name whole-cell current.
Desensitization of the whole-cell current describes the “exponential decay” of the current response, after reaching peak amplitude, to a steady level in the continuous presence of agonist. While it is believed that desensitization of glutamate receptors, along with their kinetic modulation, are important in the functional regulation of these receptors, there seems to be a disagreement regarding the role of GABA_A receptor desensitization. On one hand, there is evidence supporting the desensitization of GABA_A receptor (Leidenheimer, 1991). It has also been shown that the desensitization can be modulated by calcium/calmodulin-dependent protein kinase (Wang et al., 1995). On the other hand, at GABA_A synapses, desensitization of the receptors appears to play little role in shaping the time course of IPSCs (Borst, et al., 1994). The kinetic scheme published by R.L. MacDonald’s group represents one of the first models, in which a densensitized state (state D in Fig.3.1) has been considered for the receptor. To test the role of the state D, the Monte Carlo simulation scheme presented above was used to simulate the responses of GABA_A receptors with/without the state D to the constant presence of GABA.

Shown in Fig.4.13 are the simulated responses of 1600 GABA_A receptors when they are exposed to 100μM of GABA, starting at t=0. It is surprising to observe that the conductance responses did not show the exponential decay as expected. Furthermore, there is little difference in the responses before and after the removal of the state D from the receptor kinetic model in simulation. Another interesting observation is that the number of double-bound open channels and the double-bound receptors represent over 95% and 80% of total open and bound receptors, respectively. This dominance is achieved in a few milliseconds following initiation of the concentration clamp (Fig.4.14). This finding is in contrast to the comparable contributions from
both single- and double-bound receptors to the overall response in the simulation of simultaneous release of GABAs.

After a closer look at the kinetic scheme one notes that the transition rate from A2R to D (4/s) is about twenty times smaller than that from D to A2R (90/s) (for a ratio of 4/90 = 1/22.5 to be exact). This means that it is a lot easier for the receptor to get out of the desensitized state D than to get into it. Therefore, the lack of desensitization in conductance responses may lie in the GABA$_A$ receptor kinetic model. To further prove this point, the transition rates between states D and A2R were switched (i.e. the rate from D to A2R is now 4/s and the rate from A4R to D is 90/s, for a ratio of 90/4 = 22.5/1) and the conductance responses were simulated again under the same conditions as described above.
Figure 4.15: Time courses of the simulated conductances (top) and the number of desensitized receptors (bottom) before (solid) and after (dashed) an approximate 500 fold change in the ratio of the transition rates to and from the state D (see text for details). Other conditions are the same as in Fig3.13.
The results are illustrated in Fig. 4.15. It is obvious that the desensitization of response indeed occurred in this case. Moreover, the desensitization also resulted in the reduction in the peak response. From the time courses of desensitized GABA receptors before and after the switch of the rate constants (Fig. 4.15, bottom), one can see that, when it is about twenty times easier to get into the desensitized state than to get out of it, about 34% of GABA receptors are desensitized at the peak response. At the steady level of the conductance response, over 80% of the receptors are in the desensitized state. These results indicate that the ratio of rate constants of transition to and from the state D is very important in determining the role of the state in shaping the synaptic responses.

So far the simulations concerning the presumed desensitized state D are done under the condition of continuous concentration clamp. To test if the state D with the modified transition rates play a role in synaptic transmission, the responses to the 1 ms GABA clamp (1 mM) of 400 GABARs with the modified rate constants was simulated and compared with that before the modification (or before “switch”, as described above, Fig. 4.16). A consistently smaller response both at the peak and in the falling phase is obtained for the receptors with the modified state D. This is reasonable since the probability for receptor to stay in D is increased after the modification, which reduces the proportion of receptors in open state. It is also of interest to note that the short switches in the number of open channels is also decreased (comparing Fig. 4.10, bottom and Fig. 4.16, bottom). This finding indicates that modulation of the kinetic transition rates for the desensitization state D can change synaptic response variance.
Figure 4.16: Comparison of the responses (top) to 1 ms GABA clamp (1 mM) of the 400 GABARs before (dashed) and after the modification of state D. Time courses of the open channels in the case of modified state D are shown at the bottom. $\Delta t=0.1 \ \mu s$ and data sampling rate is 0.1 ms/point. n=20
120

Figure 4.17: Top: Superimposed responses of 400 GABARs with (dashed) without (solid) the 6 intraburst closed states to GABA clamp (1 mM for 1 ms); Bottom: Time course of single- or double-bound open channels when 400 GABARs do not have the intraburst closed states. $\Delta t = 0.1 \mu s$ and n=20.

b. Intraburst closed states

In the GABAA receptor kinetic model, there is a group of 6 closed states, namely intraburst closed states, which transit to and from the three open states (Fig.4.1). These states are believed to be short-lived since the rates of transiting to them range from 1/25th to 1/8th of the rates of transiting away from them. The following simulations are designed to examine the effect of these intraburst states. Simulated were synaptic responses of 400 GABARs to the 1 mM GABA clamp (for 1 ms) before and after removal of the 6 intraburst closed states from the kinetic model. Shown in Fig.4.17 is a comparison of the conductance responses.

Clearly, the response of the receptors without the intraburst states is “slightly”
larger, but the peaks are not statistically different (t-test). It seems that the 6 intraburst states do not have too much influence on the mean response in the falling phase. They do, however, play a role in the conductance variance especially in the falling phase of the response. This is evident by comparing the time courses of open channels before (Fig.4.10, bottom) and after (Fig.4.17, bottom) the intraburst closed states are removed in the simulations. The short switches in the number of open channels is greatly reduced after the removal of the states. Further validation of this claim can be found in the next subsection (Fig.4.19).
Effect of receptor distribution on synaptic transmission

One of the questions electrophysiologists are interested in answering is the effect of receptor distribution on synaptic responses. In the majority of the Monte Carlo simulation studies discussed above, the GABA receptors are distributed uniformly around the geometric center (on-center) of the postsynaptic membrane and GABA molecules are released from the geometric center of the presynaptic membrane. To study the role of receptor distribution, assume that there are 400 GABA receptors located on a 200 x 200 nm square patch off-center, occupying one square corner of the postsynaptic membrane, and that the site of GABA release is still at the geometric center of the presynaptic membrane. The mean response and standard deviation (sd, n=20) of the 400 GABARs to the release of 5000 GABAs are illustrated in Fig.4.18.

As expected, the peak response (154.25 pS at 2.9 ms) of the off-center GABA receptors is decreased (less than 50% of that of the on-center GABARs). The standard deviation (sd) of the membrane conductance is persistently high in the falling phase of the response. This is in agreement with the results from simulation study of the Ach synapse. In addition, it appears that the short-lived transitions (labelled with "*" in Fig.4.18, bottom), presumably due to the transitions between open states and the intraburst states, contribute at least in part to the variance of the response. This is further supported by the simulated response in which the intraburst states are removed (Fig.4.19). Clearly, the number of short-lived transitions are reduced (Fig.4.19, bottom) and the remainings do not seem to contribute to sustaining of high variance during the falling phase of the conductance response (compare Fig.4.19 top and bottom). Furthermore, no significant difference exists between the peak responses simulated before and after the removal of the intraburst states (t-test).
Figure 4.19: Time courses of conductance response and standard deviation (sd) (top) and open channels (bottom) of 400 “off center” GABARs simulated under the same condition as in Fig.18, except that the intraburst closed states are removed. A comparison is made between the simulated response before (with i, dots) and after (without i, solid) the removal. n=20.
This observation further supports the idea that the intraburst closed states do not significantly affect the inhibitory transmission in living GABAergic synapses.

**Discussion and Conclusion**

This simulation study was the first attempt to simulate GABA$_A$-receptor-mediated synaptic transmission using Monte Carlo analysis. Rather than trying to reproduce specific experimental results, the focus of the simulations was on answering some of questions of great interest in electrophysiological studies of GABAergic neurotransmission. Since the majority of the parameters in the simulations come from experimental observations, the results of the simulations will help to predict the roles of such factors as the timing of the release of vesicles, the number and distribution of available receptors and desensitization state in the shaping synaptic responses.

**Concentration of GABA and increase in available receptors**

The simulations of simultaneous release of 5000 or 50000 GABAs, which are roughly the number of GABAs in one or ten vesicles and equivalent to an average concentration of 2.5 or 25 mM in the model synapse, indicate that the majority (over 90%) of the GABAs are out of the synaptic junction in tens of microseconds following their release. Even when 50000 GABAs are released, at least one of the two parameters, receptor saturation level and GABA efficacy, is still below the experimentally predicted value. Furthermore, if pools of GABA are released simultaneously, the increasing the number of available receptors on the postsynaptic membrane does not seem to be an efficient way to enhance synaptic response.

More studies should be done to clarify the time courses of GABA release. One
thing is clear, however, from the simulation studies, that in order to achieve the predicted receptor saturation level and GABA efficacy in living GABA\textsubscript{A} synapse, there must be time intervals (not more than tens of microseconds) between the release of different pools of GABA vesicles. The average GABA concentration in the synaptic cleft is maintained at a high level by the successive release of GABA pools. In fact, it has been suggested that during mIPSCs the concentration of GABA in the synaptic cleft might surpass 0.5-1 mM for a duration that is longer than the on-rate of synaptic GABA\textsubscript{A} receptors (Maconochie et. al. 1994).

The simulation studies designed to test the hypothesis of short lasting high GABA concentration revealed that maintenance of 1 mM GABA for 1 ms in the synaptic cleft makes it possible to achieve both the receptor saturation level and GABA efficacy with a relatively small number of receptors. More importantly, the short persistence of GABA molecules enhances the efficiency of cells to increase the synaptic transmission (through increasing the number of free receptors). Specifically, a nearly 300% enhancement in peak response is achieved for a 3-fold of increase in the number of available receptors. In contrast, if no mechanism exists to keep the transmitter concentration high (i.e. 90% of simultaneous released GABAs diffuse out of synaptic junction within tens of microseconds), then only a 60% enhancement in the peak response can be obtained for a 3-fold increase in the number of postsynaptic receptors. These findings have a significant implication in the physiological (or pathological) conditions where an increase in the number of available receptors is found. For example, a nearly doubling of the number of activated functional postsynaptic GABA\textsubscript{A} receptors without any change in single-channel conductance or kinetics was demonstrated in granual cells of epileptic rats (Otis, et al., 1994), where significantly
larger GABA responses (compared with control rat) were recorded.

**Desensitized state and intraburst closed states**

In the second part of the Monte Carlo simulation studies, we first tested the roles of the desensitized state in shaping whole-cell responses. The results indicated that the state D in the GABA<sub>A</sub> kinetic model of mouse spinal neurons proposed by Twyman, et al (1992) does not induce the exponential decay of responses in the presence of a constant concentration of GABA. However, the change in transition rates, which make it easier to get into the state D than to get out of it, is capable of producing the experimentally observed desensitization of GABAergic responses. This suggests that any modulation in the physiological condition of the kinetic constants transiting to and from the state D will result in the desensitization of response. In fact, a drug-induced change in the degree of desensitization has been reported for the whole-cell responses in various experimental environments (Farb, 1990; Leidenheimer, 1991; Wang, 1995).

The fact that the kinetic model of GABA<sub>A</sub> receptor channel in mouse spinal neuron does not produce the desensitization of simulated whole-cell response makes one suspect the validity of the the model in describing the desensitization. From a different perspective, however, if the model truely reflects the kinetic transitions occuring at the GABA<sub>A</sub> receptors, it remains to be clarified, then, what contribute to the exponential decay of whole-cell currents recorded experimentally under concentration clamp.

The simulations of the responses of GABA<sub>A</sub> receptors with or without the intraburst closed states demonstrated that these states do not affect the synaptic responses
significantly. This is in agreement with the short durations of the states observed from the single channel recordings. These states do, however, contribute to the variance of the responses. They provide another intrinsic source contributing to the stochastic properties of GABA_A receptor-mediated synaptic responses.

In addition to the tests of kinetic states, simulation was done to examine how relative location between a population of GABA_A receptor channels and the site of neurotransmitter release would affect the amplitude and time course of postsynaptic response. Besides the expected reduction in the peak response, when GABA_A receptors are distributed off center in relation to the central release of neurotransmitters from the presynaptic membrane, the persistence of a high level variance during the falling phase of simulated response was also found. This is consistent with the other simulation studies of other ligand-gated receptor-mediated synaptic response (Faber, et al., 1992).

Finally, the results of both parts of simulations indicate that the higher (and longer duration) the concentration of GABA in the synaptic junction, the greater proportion the double-bound GABA_A channels represent among open channels. This suggests the ability of GABA_A receptors to amplify the presynaptic signal (i.e., the release of neurotransmitters from presynaptic terminals) since the two double-bound open states of GABA_A receptor have a higher conductance level than the single-bound one.

Monte Carlo method in simulation of synaptic transmission

The Monte Carlo simulation scheme presented in this dissertation provides a realistic way to simulate the synaptic responses in the CNS. Compared with the sim-
ulation using deterministic differential equation approach, it offers a few advantages. First of all, discretization of the postsynaptic membrane into small receptor squares makes possible the simulation of the response of receptors distributed on a geometrically unidealized membrane. Furthermore, since in the Monte Carlo method the focus is on individual transmitter molecules and receptors, it is possible to test the effect on the overall response of a subpopulation of receptors with a different characteristics from others', which is very difficult to do using differential equations. The stochastic nature of the Monte Carlo analysis closely reflects the experimentally recorded PSCs and has been utilized to study the sources of variance in synaptic responses. Finally, the Monte Carlo method simplifies the programming work in simulation of synaptic responses recorded using complicated experimental protocols. In addition, the addition or deletion of a state in a kinetic model can be easily programmed.

The obvious disadvantage associated with Monte Carlo method is the computational cost. The high cost in computational time is due not only to the requirement that a small enough time step has to be chosen but also to the need that the states of each and every neurotransmitter and receptor have to be randomly determined in each step. Moreover, in Monte Carlo analysis the attempt to improve accuracy of simulation through averaging is of high cost in computation time due to the Poisson nature of the method in simulation of diffusion. Averaging of results from $n$ runs only reduces the error by a factor of $\sqrt{n}$.

In summary, the results from the Monte Carlo simulation study of GABA$_A$ receptor-mediated responses support the recent suggestions that the concentration of GABA at GABA$_A$ synapses might be in the millimolar range and that desensitiza-
tion state does not seem to affect the amplitude of mIPSPs. The short persistence of a high concentration of GABA in the synaptic junction through consecutive release of different pools of GABAs improves the efficacy of the inhibitory neurotransmission and the efficiency of long-term potentiation of GABAergic inhibition through increasing the number of available receptors. These results will certainly help to understand the roles of different factors in the synaptic junction in shaping the response of postsynaptic neurons. Furthermore, they are also important in the explanation of physiological and pharmacological modulations of synaptic transmission at the GABA_A synapse.
GENERAL DISCUSSION

The present study, using whole-cell patch-clamp technique, indicates that GABA$_A$-receptor-mediated response in spinal DH neurons can be modulated by $\mu$-opioid receptor agonists and CaM-KII. Results of the study are discussed here in regards to the current knowledge of opioid and GABAergic pathways, synaptic plasticity, and pain modulation in spinal cord.

Opioid and GABAergic Pathways

Sites of opioid actions

Intrathecal administration of endogenous opioids or exogenous opiate alkaloids has been shown to induce behavioral analgesia (for review see Yaksh, 1993). The mechanism of the analgesia is of great interest since such information can be potentially exploited for the precise clinical control over the use of such drugs. The opioid- and opiate-induced effects are presumed to result from actions at spinal opioid receptors normally innervated by enkephalin and possibly by dynorphinergic neurons (Basbaum, 1985; Morris and Herz 1987).

The superficial laminae I-III of the spinal cord are believed to play a important role in the processing of sensory information, including nociception (Willis and Coggeshall, 1991). Consistent with this idea is the rich distribution of various opi-
oid receptors, $\mu$, $\delta$, and $\kappa$ (Morris and Herz, 1987; Stevens et al., 1991) and opioid-containing neurons and terminals in this area (Glazer and Basbaum, 1981; Ruda et al., 1984; Carlton and Hayes, 1989). The sites of opioid actions can be presynaptic or postsynaptic. Presynaptically, activation of $\mu$- and $\delta$ receptors inhibits the release of SP (Yaksh, 1993) and excitatory amino acids, glutamate and aspartate (Kangrga and Randic, 1991). The decrease in the neurotransmitter release has been shown to reduce mEPSCs (Hori et al., 1992) in the spinal DH. Anatomical (Ruda et al., 1984; Basbaum, 1985) and functional studies also suggest that the analgesic action of intrathecally applied opioids results, at least in part, from a direct inhibition of nocireceptive projection neurons. In both trigeminal (Chen and Huang, 1991) and spinal DH (Rusin and Randic, 1991) neurons, activation of $\mu$-opioid receptors has been found to modulate postsynaptic NMDA receptor-mediated responses. The present finding that the GABA$A$ receptor-mediated responses in the isolated spinal DH neurons can be modulated by $\mu$-opioids provides new evidence to support a role for opioids at postsynaptic sites.

**Interactions between opioid and GABAergic systems**

There is good evidence that GABAergic inhibitory pathways are also involved in antinociceptive actions in the spinal cord. Intrathecal application of baclofen, an analogue of GABA with high affinity for GABA$B$ receptors, produces analgesia in various animals (Wilson and Yaksh, 1978). This is in agreement with the observation that the firing of the spinal nociceptive neurons is profoundly inhibited by baclofen (Henry, 1982). These results seem to support the common believe that activation of GABA$B$ sites is responsible for GABAergic analgesic actions. However, recent
work has also shown the involvement of GABA$_A$ sites. Muscimol, which preferentially binds to the GABA$_A$ receptor, was found to produce the bicuculline-sensitive inhibition of C-fiber-mediated responses in various isolated spinal preparations (Otsuka and Yanagisawa, 1990). Furthermore, the intrathecal administration of picrotoxin and bicuculline, both of which are GABA$_A$ receptor antagonists, attenuates the pentobarbital-induced inhibition of a spinal nociceptive reflex (Stein et al., 1991).

Although opioid and GABAergic systems have both been implicated in the analgesic actions, what is not clear is the way and the extent to which these two systems interact in the spinal cord. There are data showing that opioid systems are modulated by GABAergic actions. Chronic GABAergic action was found to modulate the expression of proenkephalin mRNA in guinea pig hypothalamus (Mithell et al., 1992). At behavioral level, benzodiazepines and barbiturates, both of which are allosteric modulators of GABA$_A$ receptors, produce either an antagonism (Daghero et al., 1990; Wang and Fujimoto, 1992) or a synergistic enhancement (Yanez et al., 1990) of morphine-induced antinociception. These observations can be explained by the anatomical discovery that in lamina I, II, V and X, many GABA-immunoreactive terminals are presynaptic to enkephalin-immunoreactive cell bodies and dendrites (Liu et al., 1992). This indicates that the enkephalin regulatory mechanism is under GABAergic control. A condition which increases GABAergic tone would, hence, have a dual effect, increased inhibition of the projection neuron through direct GABA-mediated action and facilitation (or even excitation) of the projection neuron via a disinhibition of the opioid regulation. The precise level at which the nociceptive threshold is set would, of course, depend on the magnitude of activity at both the opioid and GABAergic terminals.
The other side of the story concerning the interaction between the two systems is the effects of opioid pathways on GABAergic inhibitory synaptic transmission. There are relatively fewer studies done on this aspect. Evidence does exist, however, for the presynaptic inhibition of GABA release, which in turn decreases the GABA$_A$ and/or GABA$_B$ receptor-mediated responses in granule cells and CA1 pyramidal cells of the hippocampus (Xie et al., 1992; Lupica 1995). In the latter study, analogues of cAMP failed to alter the the inhibitory effects of $\delta$- and $\mu$-opioids on the amplitude and frequency of spontaneous IPSCs, suggesting the presynaptic inhibition of GABA release is independent of PKA pathway. The present data that the activation of $\mu$-opioid receptors modulates the postsynaptic GABA$_A$ receptor sensitivity provides, for the first time, the evidence to support the idea that GABAergic inhibitory transmission is subject to the postsynaptic regulation by opioids. This observation suggests that the $\mu$-opioids (also likely, the $\delta$-opioids) in the spinal DH might exert their antinociceptive actions at least in part through modulating the postsynaptic GABA$_A$ receptor sensitivity. Consistent with this observation is the colocalization of GABA and [Met]enkephalin in the majority of enkaphalin-immunoreactive neurons in the superficial laminae of the spinal cord (Todd et al., 1992). In addition, a recent behavioral study indicates that, DPDPE, a $\delta$-preferring analogue of enkephalin, administered intracerebroventricularly in the mouse produces an antinociceptive action through mediation of spinal GABA$_A$ and GABA$_B$ receptors (Holmes and Fujimoto, 1994).

Taken together, evidence so far suggests that the interactions between opioid and GABAergic systems in the spinal cord is not only common, but also bidirectional. The interactions working together provide additional modes of regulation of the efficacy
of the synaptic transmission in the spinal DH, including nociception.

**Involvement of cAMP-dependent second messenger system in the potentiation of GABA<sub>A</sub> response by µ-opioid agonists**

Considerable evidence exists for direct regulation of GABA<sub>A</sub> receptor function by protein phosphorylation (see Macdonald and Olsen, 1994 for review). A basal level of phosphorylation of either the GABA<sub>A</sub> receptor itself, or a closely associated protein, appears to be necessary to maintain GABA<sub>A</sub> receptor function (Chen et al., 1990). In addition, GABA<sub>A</sub> receptors are substrates of a number of different serine/threonine protein kinases that are regulated by distinct second messengers. The α subunit family and subunits appear to be the major sites of phosphorylation for these kinases (Moss et al., 1992; Raymond et al., 1993; Krishek et al., 1994; McDonald and Moss, 1994). However, the functional effects observed have been complex, and sometimes even contradictory. For example, protein kinase A (PKA) has been shown to decrease GABA-induced currents in cultured spinal (Porter et al., 1990) and sympathetic ganglion neurons (Moss et al., 1992) but to increase them in cerebellar Purkinje cells (Sessler et al., 1989; Kano and Konnerth, 1992; Cheun and Yeh, 1993) and retinal bipolar and ganglion cells (Veruki and Yeh, 1992). Moreover, in dissociated CA1 hippocampal pyramidal neurons, 8-Br-cAMP produces concentration-dependent effects on GABA<sub>A</sub> currents; at nanomolar concentrations GABA currents were potentiated, whereas at micromolar concentrations they were reduced (Stelzer, 1992). Another study found no effect of PKA on GABA responses in cultured spinal neurons (Ticku and Mehta, 1990). These different effects are thought to be due to different receptor subunit combinations in the various preparations, or
to the indirect effects of phosphorylation on other regulatory proteins. Studies with recombinant and native neuronal GABA<sub>A</sub> receptors showed that a major feature of PKA phosphorylation is a negative modulation of receptor function (Moss et al., 1992; Krishek et al., 1994). The degree of negative modulation of current amplitudes and the regulation of rapid desensitization appear to be dependent on the subunit composition of the expressed GABA<sub>A</sub> receptors (Moss et al., 1992; Krishek et al., 1994).

Almost all known opioid receptors are coupled to G proteins (Childers, 1993). Like most of the G-protein coupled receptors, the effector systems for opioid receptors shown so far include adenylate cyclase (Yu and Saddee, 1986; Konkoy and Childers, 1989; Chen et al., 1988), calcium and potassium conductances (North et al., 1987; Gross et al., 1990) and possibly phospholipase C (Periyasamy and Hoss, 1990). All three major types of opioid receptors, μ, δ and κ, are predominantly coupled to pertussis toxin-sensitive (PTX) G<sub>i</sub>- or G<sub>o</sub> proteins, except in dorsal root ganglion where activation of G<sub>α</sub> protein might be involved in the opioid-induced prolongation of action potentials (Chen et al., 1988). The coupling between the opioid receptors and G<sub>i</sub> and/or G<sub>o</sub> proteins increases potassium and decreases calcium conductances in a variety of cell types including spinal neurons (North et al., 1987; Gross et al., 1990). The change in the ionic conductances have been shown to play a role in both the presynaptic and postsynaptic inhibitions of the substantia gelatinosa neurons (Yoshimura and North, 1983; Grudt and William, 1993, 1994). However, not all the function of opioids are through changes in the ionic conductances. In the rat hippocampus CA3 region, μ-opioid receptor preferring agonist FK33-824 has been found to induce the G<sub>i</sub> protein-mediated inhibition of GABA release independent of
Of all the effector systems which have been implicated with opioid receptors, the best studied is opioid inhibition of adenylate cyclase in cell lines and in brain cell membranes. In human neuroblastoma cell line (Yu and Sadee, 1986; Yu et al., 1990), µ-opioid agonists including morphine potently inhibited adenylate cyclase. In guinea pig cerebellum membranes, dynorphin was also found to inhibit adenylate cyclase (Konnkoy and Childers, 1989). The biological roles of the opioid-induced inhibition of adenylate cyclase is of a great interest due to the possible involvement of the cAMP-dependent second messenger system in the actions of opioids. The evidence so far does not support a role for the second messenger system in the presynaptic inhibition of transmitter release (Gross et al., 1990; Lupica et al., 1995). However, Results from a number of studies support an inhibitory effect of µ and δ opioids on the synthesis of cAMP (Yu et al, 1990; Wang et al, 1993) and a role for this effect in their biological actions (Crain et al., 1986; Chen et al, 1988). In mouse spinal cord-ganglion explants, cAMP or forskolin has been shown to attenuate the depressant effect of opioids on the evoked responses in the spinal DH (Crain et al, 1986). Moreover, even at the behavioral level the intrathecal injection of membrane permeable cAMP analogues in rats reversed the µ and δ opioid-mediated analgesia (Wang et al., 1993).

It has been generally accepted that the immediate target of cAMP actions in cells is cAMP-dependent protein kinase (PKA)-mediated protein phosphorylation. There are several reports which linked regulation of protein phosphorylation to opioid actions. Enkephalin was found to inhibit the phosphorylation of two brain membrane...
proteins (Ehrlich, 1978). Chronic administration of morphine induced an increase in both the PKA activity and the adenylate cyclase (Nestler and Tallman, 1988; Duman et al., 1988). It has been speculated that these chronic actions of morphine are due to the mechanisms which compensate for the acute inhibition of cAMP by opioids (Childers, 1993). Our results that the enhancing effect of μ-opioids on GABA_A receptor-mediated responses can be mimicked by Rp-cAMP and blocked by Sp-cAMP are consistent with this line of evidence, and with the negative modulation of GABA_A receptor currents shown in a broad spectrum of cells. In addition, blockade of the potentiating action of μ-opioids by PTX demonstrated in our experiments further suggests that the opioid effect on GABA_A receptor-evoked responses might have involved G_i or G_o protein-mediated inhibition of the cAMP-dependent second messenger system.

GABA_A Receptor, CaM-KII and Synaptic Plasticity

CaM-KII-mediated modulation of GABA_A receptor responses

The evidence for the direct phosphorylation of GABA_A receptors is compelling. Molecular studies have implicated PKA, PKC, tyrosin kinase and cGMP-dependent protein kinase in the modulation of GABA_A receptors through direct phosphorylation (see MacDonald and Olsen, 1994 for review). The functional effects of the GABA_A receptor phosphorylation have also been shown in a variety of cell types (MacDonald and Olsen, 1994).

Our results that the activated α-subunit of CaM-KII enhanced GABA_A receptor-mediated currents and IPSPs while heat-denatured CaM-KII did not alter the responses in isolated spinal DH neurons or in the hippocampus slices supports a role
for CaM-KII in the functional modulation of GABA<sub>A</sub> receptor. This effect of CaM-KII might have occurred as a result of direct phosphorylation of GABA<sub>A</sub> receptors since recent molecular studies found that the intracellular domains of GABA<sub>A</sub> receptor subunits contain consensus sites for CaM-KII (Machu et al, 1993; McDonald and Moss, 1994). Furthermore, anatomical studies also located both CaM-KII and GAD, the GABA synthesizing enzyme, in the spinal DH (Basbaum and Kennedy, 1986; Beson et al, 1992), where they are present in different populations of neurons. Although no colocalization of the CaM-KII and GABA was found in these distribution studies of different brain regions and the spinal cord, this does not exclude the possibility that GABA<sub>A</sub> receptor be phosphorylated by this kinase in vivo. One way in which the modulation of GABA<sub>A</sub> receptor can take place is when CaM-KII is present in a population of neurons which are postsynaptic to GABAergic terminals, express GABA<sub>A</sub> receptor but do not synthesize GABA themselves. This possibility is supported by a histochemical study of the CaM-KII and GAD distribution in monkey basal ganglia, thalamus and hypothalamus (Benson et al, 1991). In addition, our finding that GABA<sub>A</sub>-mediated responses can be enhanced by calyculin A, which increases the endogenous CaM-KII activity, further suggests a role for CaM-KII in the regulation of GABA<sub>A</sub> function in vivo.

CaM-KII is a multifunctional enzyme. A diverse group of proteins have been shown phosphorylated by CaM-KII, this including cyclic nucleotides, phosphodiesterases, calcineurin, and microtubule-associated proteins (Hanson and Schulman, 1992). Therefore, the modulation of GABA<sub>A</sub> receptor-mediated currents by CaM-KII observed in our experiments might have arisen in part (or in its entirety) from the phosphorylation of the nucleotides or proteins, which in turn may modulate GABA<sub>A</sub>
channel sensitivity. In fact, calcineurin or phosphatase 2B has been suggested as the endogenous phosphatase which is involved in the reduction of the basal level GABA_A receptor phosphorylation and GABA_A receptor “run-down” (Chen et al, 1990). The possible phosphorylation of calcineurin by CaM-KII should then have a profound, indirect effect on GABA_A receptor. In a related study on glycine receptor, gephyrin and tubulin, both of which are associated with glycine receptor, were phosphorylated by an endogenous kinase (Langosch, 1992). In addition to the possible phosphorylation of GABA_A receptor-associated proteins and phosphatase, CaM-KII might have affected the GABA_A channel activity through its actions (directly or indirectly) at allosteric modulatory sites. GABA_A receptor contains a variety of modulatory sites that are sensitive to clinically-important agents such as benzodiazepines and barbiturates (MacDaonald and Olsen, 1994).

**Role of CaM-KII-mediated modulation of GABAergic response in synaptic plasticity?**

The LTP and LTD of excitatory synaptic transmission are of great interest in neuroscience research due to their possible relevance to memory and learning. So far, the long-term modulation of synaptic efficacy has been shown in hippocampus, cerebellum and neocortex (Bliss and Lomo, 1973; Ito et al, 1982; Artola et al, 1990). A recent study indicates that distinct and long-lasting modulation can also be induced at the synaptic contacts formed between the primary afferents and spinal neurons in the superficial DH (Randic et al, 1993).

There is evidence to support a role for GABAergic actions in both LTP and LTD of excitatory synaptic transmission. A reduction in GABA_A receptor-mediated re-
sponse has been shown to facilitate the development of LTP (Wigstrom and Gustafsson, 1983) and heterosynaptic LTD (Tomasulo et al., 1993). The possible involvement of GABA$_B$ sites has been shown at presynaptic terminals, where the activation of GABA$_B$ autoreceptors results in the depression of GABA release. This depressant effect on GABA release might have an important permissive role in NMDA receptor activation, which is required for homosynaptic LTD (Davies et al., 1991). More recently, changes in GABAergic systems have been related to the influence of both age and prior synaptic activity on LTD induction in the Schaffer collateral pathway in the CA1 region of the rat hippocampus (Wanger and Alger, 1995). Specifically, the GABA$_A$ antagonist bicuculline did not affect LTD in the young animals, but did enhance LTD expression in slices from mature animals.

In addition to its effects on the excitatory LTP and LTD, the inhibitory synaptic transmission itself can also undergo long-lasting change. So far, both LTD and LTP of inhibitory transmission have been shown in the visual cortex (Komatsu 1994; Komatsu and Iwakiri, 1993). In the cerebellum, the activation of olivocerebellar climbing fibers produces a long-lasting “rebound potentiation” of inhibitory GABA responses in the Purkinje cells (Kano et al., 1992). This rebound potentiation of inhibitory transmission, together with the LTD of excitatory parallel fibers-Purkinje cell transmission, may contribute to the cerebellar learning. The exact mechanism of the long-lasting modulation of inhibitory GABAergic responses is still unknown. It is thought, however, that the Ca$^{2+}$ influx in response to the excitatory climbing fiber stimulation of Purkinje cells might be important in this process (Kano, 1992). Therefore, the long-lasting enhancement of GABA$_A$ receptor-mediated responses by CaM-KII, as shown in the present study, provides one possible explanation to the
LTP of GABAergic response in Purkinje cells.

Taken together, the sustained CaM-KII-induced potentiation of \textit{GABA}_A receptor-mediated response in isolated spinal DH neurons and IPSPs in the CA1 region of the hippocampus observed in the present study is consistent with the role of CaM-KII in the induction of LTP of excitatory transmission (Malinow et al., 1989). The persistent up-regulation of \textit{GABA}_A receptor sensitivity might be important in the long-lasting modulation of the inhibitory transmission and/or the modulation of LTD and LTP of excitatory transmission by GABAergic actions in both the hippocampus and spinal cord.


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APPENDIX A: MONTE CARLO INVERSION METHOD

In this Appendix, we review a well known random number generating technique, named the inversion method, for both continuous and discrete cases. If a random real number $x$ has probability density function $p(x)$. By definition, the probability distribution function is:

$$F(x) \equiv \int_{-\infty}^{x} p(x)dx$$

$F(x_0)$ gives the probability that $x$ will be less than or equal to $x_0$. The inversion method for generating the random number according to its density function is to draw a uniformly distributed number $r$ from $[0,1]$ and choose the $x$ which satisfies

$$x = F^{-1}(r)$$

where $F^{-1}(x)$ is the inverse of the distribution function. To prove that this is a correct procedure. Consider the probability that the $x$ generated according to this procedure will be in the interval $(x', x' + dx')$. According to the relation between $x$ and $r$, this probability is equal to the probability that $r$ lies between $F(x')$ and $F(x' + dx')$. Since $r$ is uniformly distributed in $[0,1]$, the probability is then:

$$F(x' + dx') - F(x') = F'(x')dx' = p(x')dx'$$

The second equality follows from the definition of $F(x)$. Therefore, the probability
density function of the random number \( x \) generated according to \( x = F^{-1}(r) \) is indeed \( p(x) \).

In the discrete case, the problem is to generate a random integer \( i \) according to the probability density function \( P(i) \), where \( P(i') \) is now the probability that \( i \) will equal \( i' \). The corresponding distribution function \( F(i) \) is defined by

\[
F(i) \equiv \sum_{i' = -\infty}^{i} P(i')
\]

and \( F(i_0) \) is evidently the probability that \( i \) will be less than or equal to \( i_0 \).

The inversion method for generating \( i \) according to \( P(i) \) is to draw a random number \( r \) from the uniform distribution in the unit interval and take for \( i \) that value which satisfies

\[
F(i - 1) < r \leq F(i) \tag{4.29}
\]

To prove that this is correct, let us calculate the probability that the resulting integer \( i \) will equal to \( i' \). This probability is just the probability that \( r \) will lie between \( F(i' - 1) \) and \( F(i') \), and this probability is

\[
F(i') - F(i' - 1) = \sum_{i'' = -\infty}^{i'} P(i'') - \sum_{i'' = -\infty}^{i' - 1} P(i'') = P(i')
\]

This proves that \( P(i) \) is indeed the probability density function for the random number \( i \) generated according to (4.29).
APPENDIX B: STATE TRANSITION RATES OF GABA\textsubscript{A} RECEPTOR

The rate constants of state transitions in the GABA\textsubscript{A} kinetic model (Fig.4.1):

<table>
<thead>
<tr>
<th>States (i,j)</th>
<th>Transition rate</th>
<th>States (i,j)</th>
<th>Transition rate</th>
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<td>90</td>
<td>11 0</td>
<td>4</td>
</tr>
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<td>1 4</td>
<td>435</td>
<td>4 1</td>
<td>5500</td>
</tr>
<tr>
<td>1 5</td>
<td>215</td>
<td>5 1</td>
<td>750</td>
</tr>
<tr>
<td>1 12</td>
<td>910</td>
<td>12 1</td>
<td>35</td>
</tr>
<tr>
<td>2 6</td>
<td>196</td>
<td>6 2</td>
<td>5500</td>
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<tr>
<td>2 7</td>
<td>97.5</td>
<td>7 2</td>
<td>750</td>
</tr>
<tr>
<td>2 11</td>
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<td>11 2</td>
<td>90</td>
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<td>8 3</td>
<td>5500</td>
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<td>750</td>
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<td>11 10</td>
<td>40</td>
</tr>
<tr>
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<td>50</td>
<td>12 11</td>
<td>10000000</td>
</tr>
<tr>
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<td>25</td>
<td>13 12</td>
<td>20000000</td>
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