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Modulation of excitatory synaptic transmission in the rat spinal dorsal horn

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Modulation of excitatory synaptic transmission in the rat spinal dorsal horn

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TABLE OF CONTENTS

LIST OF ABREVIATIONS

trans-ACPD	(±)-trans-1-aminocyclopentane-1,3-dicarboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP_3	2-amino-3-phosphonopropionate
APV	D-2-amino-5-phosphonovalerate
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CRF	Corrticotropin releasing factor
cyclic AMP	3,'5'cyclic adenosine-monophosphate
cyclic GMP	3',5'cyclic guanosine-monophosphate
DH	Dorsal horn
DRG	Dorsal root ganglion
DYN	Dynorphin
EAA	Excitatory amino acids
ENK	Enkephalin
EPSP	Excitatory postsynaptic potential
GAL	Galanin
Glu	Glutamate
GluR	Glutamate receptor
HRP	Horseradish peroxidase
IBMX	3-isobutyl-1-methyl-xanthine
KA	Kainate
mGluR	Metabotropic glutamate receptor
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline
NKA	Neurokinin A
NKB	Neurokinin B
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NS	Nociceptive specific
NT	Neurotensin
OXY	Oxytocin
PKA	Protein kinase A
PKI	Protein kinase inhibitor
PKC	Protein kinase C
QA	Quisqualate
Rn	Neuronal resistance
SP	Substance P
SS	Somatostatin
TRH	Thyrotropin releasing hormone
TTX	Tetrodotoxin
Vh	Holding potential
VIP	Vasoactive intestinal polypeptide
Vm	Membrane potential
WDR	Wide dynamic range

GENERAL INTRODUCTION

Explanation of Dissertation Format

This dissertation contains three papers preceded by a general introduction which includes experimental objectives, a background and literature review and a rationale; these are followed by a discussion and a list of references cited in the general introduction and discussion. Papers I and III represent two research papers already published, and paper II represents a manuscript accepted for publication in the Neuroscience Letters.

The dissertation contains a large part of the experimental results obtained by the author during the course of his graduate study under the supervision of Dr. Mirjana Randic.

Research Objectives

Anatomical and physiological studies have provided a relatively detailed description of the organization of afferent projections to the spinal dorsal horn. These studies have, in addition, emphasized the central role of the primary afferent synapse in the mediation of modulation of cutaneous sensory information in the dorsal horn. The finding that the spinal dorsal horn contains high levels of second messengers raised the possibility that second messengers may play a functional role in sensory transmission.

Recent studies demonstrated that second-messenger systems may alter nerve cell activity by modifying characteristics of voltage-gated channels and the release of neurotransmitters. The neuromodulation of ligand-gated channels, crucial for the control of synaptic strength, has been reported in only a few instances.

The objective of this research was to examine the possible modulation of the neuronal glutamate-gated channels by activation of second-messenger systems in the spinal dorsal horn. The specific purpose of the conducted experiments was to study the possible modulation of passive membrane properties (i.e. Vm and Rn), synaptic responses and the sensitivity of various postsynaptic glutamate receptor subtypes of DH neurons by the activation of cyclic AMP-dependent second-messenger system and by the activation of G-proteins and second-messengers coupled glutamate metabotropic receptor. The experiments used standard sharp-electrode technique for intracellular voltage recordings from <u>in vitro</u> spinal cord slice-dorsal root preparation of young rats and whole-cell voltage-clamp recordings from acutely isolated rat spinal dorsal horn neurons.

Background and Literature Review

This section briefly reviews the structural and functional organization of spinal dorsal horn, the excitatory amino acid mediated neurotransmission and the cAMP-dependent second messenger system, in order to provide a background information for the study of modulation of excitatory synaptic transmission and the sensitivity of postsynaptic

glutamate receptor subtypes in the spinal dorsal horn.

Structural and functional organization of the spinal dorsal horn

Sensory receptors and primary sensory neurons

Several types of peripheral receptors (cutaneous, muscle, visceral) (Table 1, Martin, 1991) transduce mechanical signals from the environment and the organism itself into electrical signals that are conducted by primary sensory neurons to the spinal cord (Campbell et al. 1989, Fitzgerald 1989, Martin 1991, Martin and Jessell 1991). The synaptic contacts of primary afferent fibers with heterogenous populations of spinal cord neurons represent the first stage in the CNS at which sensory information is processed and integrated.

The primary afferent neurons are unipolar with pericarya in dorsal root ganglia. On the basis of the pericaryon size, duration of somatic action potential, conduction velocity of nerve fibers, sensory modality, neurochemistry, chemosensitivity and distribution of cellular organelles they can be classified into several groups (Harper and Lawson, 1985a,b, Sugiura et al., 1988, Martin, 1991). Two principal groups have been described in dorsal root ganglion (DRG) (Liebermann, 1976, Harper and Lawson, 1985a,b). About 30-40% of neurons have large pericarya (30-70 μm in diameter) that stain with basic dyes or silver salts, short-duration action potentials (0.49-1.35 ms at the base) that are tetrodotoxin-sensitive, and give off large diameter myelinated axons (Aα or Aβ). They are classified as A type. The remaining 60-70% of neurons have smaller (25-30 μm) darkly-

staining pericaryon, long duration action potentials (0.5-8.0 ms at the base) that are in some cases tetrodotoxin-insensitive, and contain a prominent Ca^{++} component. In general they give off small diameter axons (Aδ or C) that are poorly myelinated or unmyelinated. This type is classified as B type. In addition to the two major groups there is a considerable number of intermediate size cells. Recent evidence suggests that a specific fiber type is not simply related to the cell body size so that C fibers may have large or small cell bodies (Hoheisel and Mense, 1986).

The axon of the ganglion cell often follows a highly convoluted path and then divides to form two branches. The central branch goes to the spinal cord through the dorsal root and the peripheral branch contributes to the peripheral nerve. Together they form a primary afferent fiber. The primary afferent fibers are classified on the basis of sensory modality, presence of myelin, fiber diameter, and conduction velocity. The afferent fibers from skin are alphabetically coded: Aα, Aβ, Aδ and C. Their associations with different receptor types and sensory modalities are shown in table 1 (Martin, 1991). Aα fibers have the highest conduction velocity (30-50 m/s), the largest fiber diameter and are the most myelinated whereas C fibers are the slowest (< 1.4 m/s), have the smallest fiber diameter and are unmyelinated. The classification of muscle afferents is different and is coded with roman numbers I-IV, with I being large myelinated, II small myelinated, III smaller myelinated and IV being unmyelinated fibers. Table 2 shows the classification for muscle afferents compared with classification for cutaneous sensory afferents, fiber diameter and conduction velocity (Martin, 1991).

Central processes of primary afferent fibers enter the spinal cord at the dorsal root entry zone (Fitzgerald, 1989). In general, slow conducting Aδ and C fibers travel in the lateral part of dorsal white matter including Lissauer's tract, whereas A fibers travel more medially. After entering the spinal cord they branch in the white matter and give off collaterals that terminate in the gray matter. Dorsal root afferents give off most of the collaterals in their segment of entry but the rostrocaudal spread is significant. Although the spread is relatively small (approximately one segment at midthoracic level), it involves several segments at the lumbosacral level (Chung et al., 1979). It is in general bigger for large diameter (A) than small diameter (C) fibers. The two types of fibers differ also in the pattern of termination. Aα and Aβ fibers project predominantly to deeper laminae (III-V) and Aδ and C fibers terminate in the superficial laminae (I-III) of the dorsal horn (Wolf, 1987, Fitzgerald, 1989). An interesting observation that complicates the concept of anatomical separation of afferent and efferent neurotransmission was made in the cat lumbosacral enlargement, where Coggeshall et al. (1974) demonstrated the presence of afferent fibers in the ventral root. Similar observation was made in the rat and human (Coggeshall et al., 1975, 1977) spinal cord.

In addition to their morphological and physiological differences, the dorsal root ganglion neurons differ by their chemical content (Jessell and Dodd, 1989). The immunocytochemical studies of primary sensory neurons demonstrated the existence of at least ten peptides with a possible neurotransmitter function (e.g. SP/NKA/CGRP/SS/CCK/VIP/CGRP/GAL/ENK/DYN).

Table 1. Receptor types active in various sensations (Martin, 1991)

Receptor type	Fiber group	Quality
Nociceptors		
Mechanical	Aδ	Sharp, prickling pain
Thermal and mechano-thermal	Aδ	Sharp, prickling pain
Thermal and mechano-thermal	C	Slow, burning pain
Polymodal	C	Slow, burning pain
Cutaneous and Subcutaneous mechanoreceptors		
Meissner's corpuscle	Aβ	Flutter
Pacinian corpuscle	Aβ	Vibration
Ruffini corpuscle	Aβ	Steady skin indentation
Merkel receptor	Aβ	Steady skin indentation
Hair-guard	Aβ	Flutter
Hair-tylotrich	Aβ	
Hair-down	Aβ	
Muscle and skeletal mechanoreceptors		
Muscle spindle primary	Aα	Limb proprioception
Muscle spindle secondary	Aβ	Limb proprioception
Joint capsule mechanoreceptors	Aβ	Joint capsule pressure;limited role in limb proprioception
Golgi tendon organ	Aα	

Table 2. Afferent fiber groups (Martin, 1991)

	Muscle nerve	Cutaneous nerve	Fiber diameter (μm)	Conduction velocity (m/s)
Myelinated				
Large	I	A-C	13-20	80-120
Small	II	Aβ	6-12	35-75
Smallest	III	Aδ	1-5	5-30
Unmyelinated	IV	C	0.2-1.5	0.5-2

The same studies provided evidence for the co-existence of several peptides in a single primary sensory neuron (Lee et al., 1985, Jessell and Dodd, 1989, Seybold et al., 1989).

Termination of primary afferent fibers

The different classes of primary afferent fibers that convey distinct somatosensory modalities take specific routes and end in different regions of the spinal cord. By this means the specific sensory information conveyed by a particular mechanoreceptor, nociceptor or thermoreceptor in the skin is maintained within the central nervous system. Labeling studies with horseradish peroxidase have shown that the terminals of each cutaneous nerve occupy a clearly defined region with little or no overlap with the territories of nearby nerves (Koerber and Brown 1980, Molander and Grant 1986). Furthermore within the territory of a given cutaneous nerve there is a somatotopic arrangement of terminals, thus for instance each digit has its own area of termination within the tibial field (Molander and Grant, 1985). Unlike cutaneous nerves, muscle and visceral nerve terminals have no clear somatotopic arrangement in the spinal cord (Molander and Grant, 1987). In general, primary nociceptive afferents have been shown to terminate in layers I and II (Light and Perl, 1979, Gobel et al., 1985). Some of these afferents give collaterals to the lamina V. Unmyelinated and polymodal nociceptive afferents (C) are considered to terminate in layer I (Gobel et al., 1981) and possibly also in layers IIa and V (Dubner and Benett, 1983). High-threshold mechanoreceptors (Aδ) terminate in layers I and IIa. Direct evidence for termination of Aδ high-threshold

mechanoreceptive afferents in the superficial laminae comes from horseradish peroxidase injections into the axons of functionally identified afferents (Light and Perl, 1979). Horseradish peroxidase filled the terminal arbors of these afferents, and its reaction product was identified in layers I and IIa with both light and electron microscopic analysis. In contrast, low-threshold mechanoreceptive primary afferents, also identified morphologically by horseradish peroxidase injections of their axons, terminate heavily in layers III to IV and sparsely in layer IIb (Brown et al., 1977, Brown, 1981). This differential distribution of nociceptive and non-nociceptive afferents at least partially accounts for the predominance of second-order nociceptive neurons in layers I and IIa and the predominance of low-threshold mechanoreceptive neurons in layers III-IV (Dubner and Bennett, 1983, Gobel, 1979). Interestingly, the somatotopic map for C Fibers in substantia gelatinosa is similar to that for A fibers in deeper laminae, but the two are not strictly in register, due to dorsoventral obliquity of the A fiber terminals (Wolf and Fitzgerald, 1986). In addition to the specificity of central termination projection each class of primary afferent fibers may possess unique arborization patterns, spacing of the collateral branches, and arrangement of buttons on the axon terminals (Cervero, 1986).

Cytoarchitecture of the dorsal horn

Current nomenclature of the cytoarchitecture of gray matter of the spinal cord is based on the Rexed's study of the cat spinal cord (Rexed 1952, 1954). He used the Nissl-staining technique to study morphological

characteristics of spinal cord neurons (i.s., the sizes, shapes, densities and distribution of neuronal somas etc.) without taking into the account the dendritic trees. Based on these observations he proposed the anatomical division of the spinal cord gray matter in 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. The importance of this laminar organization has been emphasized by the discovery that the afferent fiber classes distribute themselves to fit the laminae (Fitzgerald 1989). This division has become generally accepted (Brown, 1981, Willis and Coggeshal 1978). It has been further supported by physiological investigations and has been extrapolated to every mammalian species studied (Paxinos and Watson, 1982, Wall, 1991).

Lamina I (Marginal zone) is the most superficial layer of the dorsal horn, composed of neurons varying in size from small (5x5 to 10x10 μm) to medium and large (10-15x30-50 μm) (Rexed, 1952). Characteristic for the lamina I are the large, marginal cells (Ramon y Cajal, 1909). They have flattened cell bodies with an ellipsoid dendritic domain, which is confined to lamina I. The marginal cells project predominantly to the lateral cervical nucleus, the thalamus, the medullary and midbrain reticular formation (Brown, 1980). However, as local circuit neurons they give projections also to the spinal cord a segment or two away from the cell body (Burton and Loewy, 1976). This approximately 12-20 μm thick layer (Hunt et al.1980) is the major termination site of small myelinated afferent fibers (Aδ) from skin; the latter form synapses with dendrites of the marginal cells (Sugiura et al., 1986). In addition lamina I receives

inputs from C fibers (nociceptive) (Sugiura et al., 1986), from interneurons in laminae II and III, and some descending, presumably inhibitory neuronal elements arising from the nucleus reticularis magnocellularis (Basbaum et al., 1978). According to their physiological activation characteristics three groups of neurons have been described in this lamina: nociceptive-specific neurons (NS), wide-dynamic range neurons (WDR, which respond to both nociceptors and low threshold mechanoreceptors) and thermoreceptive neurons that respond to warming and cooling (Kniffki, 1989). The lamina I neurons differ also by their peptide content. At least ten neuropeptides are localized in the lamina I (e.g. SP, NKA, CCK, SRIF, VIP, NT, ENK, NPY, DYN and GAL) (Jessel and Dodd, 1989).

Lamina II (Substantia gelatinosa of Rolando) is an easily distinguishable translucent area ventral to lamina I. Its pale appearance is due to absence of myelinated fibers and densely packed small cells (Ramon y Cajal, 1909, Rexed, 1952). Based on the dendritic arborizations and axonal projections of its neurons, lamina II is further subdivided in two regions. The outer region (lamina IIo) is 30-40μm thick, and the inner region (lamina IIi) is 40-50 μm thick (Brown, 1981). Two types of neurons are present in lamina II. The larger (16-22μm) stalked cells with cone-shaped dendritic domains are predominantly found in the lamina IIo and smaller (5x5 - 10x10μm) islet cells with longitudinally oriented dendritic trees are mostly found in lamina IIi. Functionaly, the neurons in the lamina IIo are predominantly NS or WDR with axons projecting into lamina I (Sugiura, 1975, Brown, 1981), while the neurons of lamina IIi respond only to innocuous mechanical stimuli (Brown, 1981). The input to the lamina II

is largely from unmyelinated and smaller myelinated fibers. Unmyelinated (C) fibers predominantly terminate in this area while smaller myelinated (Aδ) fibers terminate in this as well as in lamina I. There is almost no input from large myelinated fibers (Aβ). Fourteen different peptides with potential neurotransmitter function were identified in this lamina (SP, NKA, VAS, OXY, CCK, SRIF, VIP, NT, ENK, NPY, DYN, GAL, CGRP and TRH) (Jessel and Dodd, 1989).

Rexed's laminae I and II comprise the superficial dorsal horn (Cervero, 1989). Because of the nociceptive nature of the smaller myelinated and unmyelinated fibers terminating in the laminae I and II, the superficial dorsal horn is considered to be an important site for transmission and modulation of nociceptive information.

Lamina III is relatively broad and limited medially by white matter and laterally by substantia gelatinosa. It is composed of small size neurons (7x10 - 8x12 μm), that are less densely packed than in lamina II and contain some myelinated fibers (Rexed, 1952, Brown, 1981). Input in the lamina III is predominantly from low threshold mechanoreceptive fibers (Aβ) and from proprioceptive fibers (Szentagothai, 1964). The peptides are less abundant in this area (SP, ENK, NT and TRH).

Lamina IV is thicker than the previous laminae, medially limited by white matter and laterally by more superficial areas. The distribution of cells is less dense and there are more fibers than in lamina III, which makes it look darker (Rexed, 1952, Brown, 1981). Characteristic of lamina IV is neuronal heterogeneity, with neurons varying in size from smaller (8-11μm) to large(35-45μm). The most prominent are large star-shaped cells

with long dendrites. Dorsally-directed dendrites penetrate the substantia gelatinosa and may receive input from substantia gelatinosa neurons (Szentagothai, 1964). Lamina IV neurons project centrally through the spinothalamic, spinocervical or propriospinal tract (Willis and Coggeshall, 1978). The neuropeptide content is similar to the peptide content of lamina III.

Lamina V is located in the neck of the dorsal horn, medially limited by white matter and laterally transformed gradually through a mesh of myelinated fibers into white matter. The neurons show even more variability than in the lamina IV (8x10μm to 30x40μm). Their projections are similar to the ones of lamina IV and the inputs are predominantly C fibers from viscera, Aδ from skin and group IV from muscle. This lamina is richer in neuropeptide content than lamina IV and contains at least eight potential peptide neuromodulators (SP, CCK, SRIF, CRF, ENK, NPY, DYN and GAL)

Lamina VI exists only in cervical and lumbar enlargement of the spinal cord. The cells in this area are smaller (8x8μm to 30x35μm) and more regular in shape than cells in lamina V. The neurons have complex afferent input, with a large proportion of neurons responding to low-threshold muscle mechanoreceptors (Wall, 1989). The neuropeptide content is similar to the previous area.

Laminae II, IV, V and VI comprise the nucleus proprius, which integrates sensory input with information that descends from the brain and the region of the base of the dorsal horn where many of the neurons that project to the brain stem are located.

Fast excitatory neurotransmission in the spinal dorsal horn

Experimental evidence indicates that there are two major classes of chemical compounds that are released during activation of primary afferent fibers in the mammalian spinal DH. Dicarboxylic amino acids, glutamate (Glu) and aspartate are the major candidates for the fast excitatory neurotransmitters (Mayer and Westbrook 1987, Kangrga et al., 1988, Gerber et al., 1989a, Yoshimura and Nishi, 1993) whereas tachykinins (substance P, neurokinin A) appear to be functionally involved in the slow excitatory synaptic transmission (Urban and Randic, 1984).

Excitatory amino acids (EAA)

It is a widely accepted idea that glutamate, or a related amino acid, is the major primary afferent neurotransmitter mediating fast excitatory transmission in the mammalian brain (Mayer and Westbrook, 1987; Wroblewski and Danysz, 1988; Watkins, 1990), and spinal cord (Jahr and Jessell, 1985, 1987, Kangrga et al., 1988, Gerber et al., 1989a, Yoshimura et al., 1990, 1991, Yoshimura and Nishi, 1993). It is localized in dorsal root ganglion neurons (DeBiasi and Rustioni, 1988, Henley et al., 1993) and released upon electrical activation of primary afferent fibers or other types of stimulation (Kangrga and Randic, 1990, 1991). The application of Glu to dorsal horn neurons induces a depolarizing response (Curtis et al., 1960; Schneider and Perl, 1985, 1988) that closely resembles the fast EPSP induced by dorsal root stimulation (Gerber and Randic, 1989c; Yoshimura and Jessel, 1990). Glutamate activates at least three major ionotropic receptor

subtypes, characterized by their selective agonists N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (KA). In addition it acts at metabotropic receptor subtypes that are activated by a selective agonist trans-ACPD, or by a non-selective agonist QA (quisqualate) (Watkins and al., 1990, Sommer and Seeburg, 1992, Schoepp and Conn, 1993).

Ionotropic glutamate receptors

Ionotropic glutamate receptors are ligand-gated ion channels, that upon activation increase permeability to Na^+, K^+ and Ca^{++} ions. The NMDA glutamate receptor is a clearly distinct entity and can be pharmacologically isolated from other ionotropic glutamate receptor subtypes by use of a specific agonist NMDA and an antagonist D-2-amino-5-phosphonovalerate (APV)(Mayer and Westbrook, 1987). The functional difference between AMPA and KA receptors is less obvious since they lack specific antagonists that can differentiate between AMPA and KA responses. The pharmacological classification in distinct categories is based on existence of high affinity binding sites for KA and AMPA with disparate anatomical distributions (Cotman et al., 1987). This distinction was supported by different single-channel conductances observed for AMPA and KA responses (Ascher and Nowak, 1988, Wyllie, 1993) and by differential distribution of AMPA and KA receptors on the dendrites of an individual DH neuron (Arancio, 1993).

The reversal potential, of ionotropic glutamate receptors (AMPA, KA and NMDA) is close to 0mV, which suggests mixed ion conductance mechanism

(Mayer and Westbrook, 1987, Gerber and Randic, 1989). Functional characteristics are the best known for NMDA-gated ion channels. The NMDA receptor has a micromolar affinity constant for glutamate (Olverman et al., 1984). The receptor-channel complex exhibits multiple conductance states (Ascher et al. 1988, Ascher and Nowak, 1988) with a main conductance state of 50pS. Relatively large conductance makes the channel permeable not only for Na^{+}, K^{+} but also for larger ions like Ca^{++} (Ascher and Novak, 1988). The NMDA receptor channel is unique among ligand gated channels. In the presence of physiological levels of Mg^{++} it exhibits a strong inward rectification. The inward rectification is due to voltage-dependent block by Mg^{++} ions (Mayer and al., 1984) and is absent when Mg^{++} is removed from the medium, or at depolarized membrane potentials. NMDA receptor is allosterically potentiated by glycine in a strychnine-insensitive manner (Johnson and Ascher, 1987) and glycine also reduces the desensitization of NMDA currents (Mayer et al., 1989). It can be noncompetitively blocked by several phencyclidine-like antagonists like MK 801 (Lodge and Johnson, 1990), and up- or down-regulated by Zn^{++} (Mayer and Westbrook, 1987, Hollmann et al., 1993). Characteristic of NMDA receptor-channel is the lengthy open state of the channel that can last several hundreds of milliseconds (Lester et al., 1990).

Non-NMDA glutamate receptors are activated by specific agonists, AMPA and KA, and blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline (NBQX)(Watkins et al., 1990, Sheardown et al., 1990). The activation of AMPA receptor-channels open rapidly inactivating (3ms) low conductance states (Ascher and Nowak,

1988) and the activation of KA receptor channels opens non-inactivating low conductance states. The predominant conductance state for AMPA-activated channel is 8pS and for KA 4pS, which makes the channels relatively impermeable for larger Ca^{++} ions. However, AMPA/KA channels with higher conductances that permeated significant amounts of Ca^{++}, were described in a subpopulation of hippocampal neurons (Iino et al., 1990). Although the non-NMDA receptor activated channels show little or no rectification kainate-activated currents exhibiting outward rectification were reported in a subpopulation of hippocampal neurons and in rat spinal trigeminal neurons (Iino et al., 1990, Gu and Huang, 1991). The observed outward rectification is due to voltage-independent Ca^{++} block of KA channel.

Recently, more than sixteen subunits of ionotropic glutamate receptor have been cloned from the rodent CNS (Hollmann et al., 1989, Gasic and Hollmann, 1992, Sommer and Seeburg, 1992) and the chromosomal localization of human glutamate receptor genes was determined (McNamara et al., 1992). The cloned GluR subunits have little sequence similarity with other ligand-gated channels; however, they share basic structural similarities like four putative membrane spanning domains, extracellularly located C and N terminals and highly conserved second transmembrane domain proposed to form the ion-conducting pore (Hollmann et al., 1989).

Currently there are four cloned AMPA subunits (GluR1-GluR4) (Hollmann et al., 1989, Boulter et al., 1990, Nakanishi et al., 1990, Keinanen et al., 1990) that show a high degree of sequence similarity. They have relatively high affinity for AMPA binding and, when expressed in oocytes, form functional AMPA channels. Each of these four proteins can exist as two

variants generated by alternative splicing (flip and flop) (Sommer et al., 1990). The differentially spliced subunits are functionally different and form ion channels which differ in their patterns of desensitization. Another functional difference, the permeability for Ca^{++} ions, also depends on the sequence of the encoding subunit and the amino acid composition of subunits in the ion channel. When expressed in *Xenopus* oocytes alone or in combination, GluR1 and GluR3 form receptor-channel complexes that have substantial Ca^{++} permeability, but, when GluR2 is coexpressed with either GluR1 or GluR3, the receptor channels have little or no Ca^{++} permeability (Hollmann et al., 1991). Further studies (Hume et al., 1991, Verdoonn et al., 1991) have demonstrated that the calcium permeability is determined by a single amino acid (arginine) within the predicted second transmembrane segment of the GluR2 subunit. A site-specific mutation of a positively charged arginine to a neutral glutamine (Q590R) in the structure of GluR2 can confer significant Ca^{++} conductance to the channel (Hume et al., 1991).

KA receptor family (GluR5, 6, 7 and KA-1, -2) (Bettler et al.,1990, Werner et al., 1991, Egebjerg et al., 1991, Bettler et al., 1992, Sommer et al., 1992) has approximately 40% sequence homology with AMPA receptors and binds KA with significantly higher affinity than AMPA (Gasic and Hollmann, 1992). Even though the functional role of various subunits is not clearly established, at least one, GluR6 forms a homomeric pore that is activated by KA and not by AMPA (Egebjerg et al., 1991). This finding provides a strong support for existence of two distinct subtypes of non-NMDA (AMPA and KA) receptor.

The currents mediated by homomeric non-NMDA receptors differ from the

non-NMDA currents seen in natural membranes. It seems that non-NMDA receptors require expression of heteromeric receptor channel complexes to obtain the features of non-NMDA receptors seen in natural membranes (Boulter et al., 1990, Nakanishi et al., 1990). A recent study has confirmed this observation by demonstrating the coexistence of five different AMPA subunits in a single Purkinje cell (Lambolez et al., 1993).

NMDA receptor subunits (NMDAR1 and NMDAR2A-NMDAR2D) (Morioshi et al., 1991, Nakanishi, 1992, Monyer et al., 1992) were cloned last and differ from other glutamate receptor subunits by large intracellular and extracellular domains (Moriyoshi et al., 1991). Additional studies, have demonstrated the site critical for control of Ca^{++} permeability and Mg^{++} blockade localized on the second transmembrane segment (Buranshev et al., 1992). Replacement of asparagine by a glutamine residue decreases calcium permeability of the channel and slightly reduces magnesium block. Interestingly, this site corresponds to the site determining the Ca^{++} permeability of AMPA receptor channels. Thus, a single amino acid position in a channel-forming region may account in part for differential behavior of NMDA and non-NMDA channels.

The validity of a general ligand-gated receptor model (proposing extracellular localization of N and C terminal) for the NMDA receptor was questioned in a recent study of the phosphorylation sites for phosphorylation by protein kinase C (Whitemore et al., 1993). Their localization on C terminal domain suggests intracellular localization of the domain and therefore different receptor model. This finding was supported by a recent study of the intracellular localization of C-terminal

antibodies (Martin et al., 1993). In contrast to non-NMDA channels, when expressed in oocytes, homomeric NMDA channels exhibited most of the features of the NMDA receptors observed in natural membranes (Moriyoshi et al., 1991).

At the primary afferent synapse, and also at most other synapses of the central nervous system the majority of fast EPSPs are mediated by non-NMDA glutamate receptors (Jahr and Jessell, 1985, Yoshimura and Jessell, 1989, Gerber and Randic, 1989a, Yoshimura and Nishi, 1993). Even though the NMDA subtype of glutamate receptor often contributes to the late component of fast EPSP (Gerber and Randic, 1989a, Hestrin et al., 1990) its most important role is the mediation of various forms of neuronal plasticity and pathology. It is required for induction of LTP in CA1 area of hippocampus (Nicoll et al., 1988, Collingridge et al., 1983, Collingridge and Singer, 1990), in striatum and in the spinal dorsal horn (Randic et al., 1993). In the spinal cord it also contributes to the generation of slow EPSP (Gerber and Randic, 1989b, Gerber et al., 1991) and underlies the wind-up phenomenon (Dickenson, 1987, 1991), that can be important in mediation of pain. Blockade of NMDA receptor has been shown to prevent the pattern formation in neural system (Udin and Scherrer, 1990) and overexcitation can lead to epileptogenesis and neurotoxicity (Daw et al., 1993, During and Spencer, 1993).

Metabotropic glutamate receptor

In addition to the ionotropic receptors that directly activate ion conductances to specific ions, glutamate acts also on metabotropic

receptors, that are coupled through G-proteins to several different second messenger systems (Miller, 1991, Baskys, 1992). The two families of glutamate receptors can be distinguished pharmacologically and differ also in their molecular structure. The metabotropic glutamate receptor is nonselectively activated by quisqualate or ibotenate and selectively activated by (±)-trans-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD) or its active enantiomer 1S,3R-ACPD (Sugiyama et al., 1989, Irving et al., 1990). Even though ACPD can modulate ionotropic Glu receptors, ACPD does not directly activate ionotropic Glu receptors. Metabotropic receptor function is blocked by 2-amino-3-phosphonopropionate (AP3) (Schoepp et al., 1990, Irving et al., 1990) or (S)-4-carboxyphenylglycine and (RS)-α-methyl-4-carboxyphenylglycine (Eaton et al., 1993) and not by the inhibitors of ionotropic Glu receptors, APV and CNQX (Shinozaki et al., 1989). The potency of the AP3 varies in different systems and AP3 has been unable to block the mGluR1-induced phosphoinositol hydrolysis (Aramori and Nakanishi, 1992), and hippocampal synaptic depression induced by 1S,3R-ACPD (Goh and Musgave, 1993). However, recent study reported that a novel selective competitive inhibitor of metabotropic receptor, (RS)-α-methyl-4-carboxyphenylglycine, is a highly potent suppressor of metabotropic receptor-dependent LTP in hippocampus, whereas AP3 was without effect (Bashir et al., 1993). The cDNA encoding a phosphoinositide-coupled mGluR was cloned by functional expression in *Xenopus* oocytes (mGluR1) (Masu et al., 1991, Houamed et al., 1991). At least four additional cDNAs were obtained recently by use of cross-hybridization technique (mGluR 2-5) (Tanabe et al., 1992). Metabotropic receptors resemble other G-protein

linked receptors and are composed of one unit containing seven putative transmembrane domains. They show, however, little sequence homology with other G-protein linked receptors (Masu et al.,1991). The characteristically large extracellular and intracellular domains, are probably responsible for a large variety of interactions of mGluR with second messenger systems. In addition to activation of phosphoinositol (Sladeczek, 1985, Sugiyama et al., 1987) second messenger pathway, metabotropic glutamate receptor can act through activation of phospholipase D and up or down-regulation of cyclic AMP-dependent second messenger system (Tanabe et al., 1992, Schoepp and Conn, 1993). *In situ* hybridization has demonstrated differential distribution of mGluR subunits in the nervous system (Masu et al., 1991, Tanabe et al., 1992, Martin et al., 1992). It is possible that the presence of different mGluR subtypes accounts for variability of effect of mGluR agonists in different experimental models.

Electrophysiologic studies have demonstrated that metabotropic receptors may play an important role in regulation of neuronal excitability and synaptic transmission in the brain (Baskys, 1992). The activation of mGluR is capable of depolarizing neurons (Zheng and Gallagher, 1991, 1992, Charpak and Gähwiler, 1991, Batchelor and Garthwaite, 1993) and increasing their excitability (Salt and Eaton, 1991) through reduction of voltage-dependent and Ca^{++}-dependent K^{+}conductances (Stratton et al., 1989, Charpak et al., 1990, McCormick and von Krosigk, 1992). However, in cerebellar granule cells (±)ACPD reduces excitability of neurons by increasing the Ca^{++}-dependent K^{+}conductance (Fagni et al., 1991). In addition to modulation of K^{+}conductances· activation of mGluR also affects voltage-

dependent Ca^{++}channels. A recent study in cultured hippocampal neurons showed the inhibitory effect of activation of metabotropic receptor on high threshold Ca^{++} conductance (Lester and Jahr, 1990). Besides modulation of passive membrane properties of nerve cells metabotropic GluR modulates also synaptic transmission. It can depress both excitatory synaptic transmission and inhibitory synaptic transmission (Hestrin et al., 1990, Baskys and Malenka, 1991, Lovinger, 1991, Swartz et al., 1993). The depression of synaptic transmission that has been observed at hippocampal synapses seems to be of presynaptic origin since responses to exogenously applied neurotransmitters are not depressed (Hestrin et al., 1990). It is likely that inhibition of Ca^{++} channels decreases the release of neurotransmitter presynaptically (Swartz et al., 1993). In contrast to these observations, recent study reported potentiation of synaptic transmission by the activation of metabotropic receptor (Bashir et al., 1993). In addition to modulation of presynaptic neurotransmitter release, mGluR can affect synaptic transmission also by modulation of postsynaptic neurotransmitter receptors. Studies in hippocampus and in oocytes injected with brain mRNA showed that the activation of mGluR can enhance the NMDA receptor responses (Aniksztejn et al., 1991, Harvey et al., 1991, Kelso et al., 1992) and can have a role in the generation of long-term potentiation in the hippocampus (Otani and Ben-Ari, 1991, McGuiness et al., 1991, Zheng and Gallagher, 1992, Bortolotto and Collingridge, 1993, Bashir et al., 1993) and long-term depression in cerebellum (Linden et al., 1991). Although AMPA receptor plays a key role in expression of both forms of synaptic plasticity trans-ACPD did not modulate AMPA responses of hippocampal neurons (Aniksztejn et

al., 1991, Harvey et al., 1991) and in nucleus tractus solitarious neurons (Glaum et al., 1993). However, recent study in hippocampus suggests potentiation of AMPA receptor responses as a possible mechanism of metabotropic glutamate receptor induced long term potentiation (Bortolotto and Collingridge, 1993).

Modulation of synaptic transmission by protein phosphorylation

Protein phosphorylation represents one of the most effective mechanisms of control of various cellular processes (Edelman et al., 1987) and is particularly important in the nervous system (Drummond, 1984, Dudai, 1987). Recent studies have demonstrated the importance of phosphorylation in regulation of synaptic transmission through actions on presynaptic release of neurotransmitter, resting membrane properties of neurons and postsynaptic receptor responsiveness (Nairn et al., 1985, Kaczmarek and Levitan, 1987, Huganir and Greengard, 1990, Greengard et al., 1993).

Protein phosphorylation involves the reversible covalent modification of hydroxyl groups of serine, threonine and tyrosine in substrate proteins by a phosphotransferase reaction. The principal chemical reaction is transfer of phosphate from ATP to substrate protein. This reaction is catalyzed by enzymes, protein kinases, which are in most cases regulated by a complex second messenger system. The introduction of a negatively charged phosphoryl group can alter the conformation of a substrate to modify the function of enzyme, a cytoskeletal protein, an ion channel or a transcriptional activator. These changes, which depend on elevated

concentrations of second messengers in the neuron usually last from seconds to minutes (Bacskai et al., 1993), much longer than, for instance, the changes in membrane potential produced by the activation of ligand-gated channels.

Several neurotransmitter receptors act through modulation of phosphorylation processes rather than by direct activation of ion channel (eg. α and β adrenergic, muscarinic ACh, metabotropic glutamate, serotoninergic, histaminergic, dopaminergic and receptors for neuropeptides and rhodopsin)(Schwartz and Kandel, 1991, Chen and Huang, 1991, Rusin et al., 1992, Bekkers, 1993). These receptors are coupled to G proteins, which in turn activate the second messenger generating enzymes. Second messengers (cyclic AMP, cyclic GMP, phosphoinositol and Ca^{++}), that are the end products of second messenger systems are small diffusible molecules . They can activate a specific protein kinase or mobilize Ca^{++} from intracellular stores, thus initiating a change that modifies the cell's biochemical state. In addition some of the second messengers (cyclic AMP, cGMP) can directly gate an ion channel (Hockberger and Swandulla, 1987, Schwartz and Kandell, 1991). The cascade of reactions allows high rate of amplification of the external stimuli and allows a complex regulation trough interaction of various signals at different steps of the cascade reaction (Edelman, et al., 1987).

The most studied protein kinases are cyclic AMP-dependent protein kinase, cGMP-dependent protein kinase, calcium/calomodulin-dependent protein kinase and protein kinase C. They are regulated through corresponding second-messenger systems and phosphorylate specific substrate

proteins. The specificity of their actions is defined by a sequence of amino acids adjacent to phosphorylation site on the substrate protein "consensus phosphorylation site" (Nestler and Greengard, 1984, Huganir and Greengard, 1990, Kennelly and Krebs, 1991). The action of kinases is balanced by the activity of phosphoprotein phosphatase, the enzyme that catalyzes the hydrolysis of covalent phosphate bond. This allows the system to maintain a steady-state phosphorylation activity, and by up or down regulation control various cellular processes.

Cyclic AMP-dependent second-messenger system

The cyclic AMP-dependant second-messenger system is one of the most studied second-messenger systems in the nervous system. The central unit of the system is the enzyme adenylate-cyclase (Casperson et al., 1987), which is regulated by stimulatory and inhibitory G-proteins coupled to membrane receptor proteins. The adenylate-cyclase catalyzes generation of cyclic AMP from ATP. Four molecules of cyclic AMP bind to two regulatory subunits of protein kinase A tetramere (Taylor et al., 1990) and cause dissociation of catalytic subunits from regulatory subunits. Catalytic subunits are the effector of the second messenger system and phosphorylate substrate proteins. The role of cyclic AMP-dependent protein kinase in modulation of presynaptic release of neurotransmitters has been well established in several different models (Greengard et al., 1972, Castelucci et al., 1980, Dudai, 1987, Hu et al., 1993). Also well understood is its role in the modulation of passive membrane properties of the nerve cells through modulation of voltage-gated channels (Klein et al., 1982, Huganir et al.,

1986, Huganir, 1987, Onozuka et al., 1988). On the other hand, the modulation of postsynaptically located ligand channels, essential for control of synaptic efficacy, has been just recently demonstrated and is still not well understood (Huganir et al., 1986, Miles and Huganir, 1988, Kirkness et al., 1989, Huganir and Grengard, 1990, Porter et al., 1990, Browing et al., 1990, Greengard et al., 1991, Wang et al., 1991).

In view of the involvement of protein kinases activation in most of neuromodulation-related phenomena, it is interesting that currently known ionotropic glutamate receptor subunits contain several consensus phosphorylation domains for phosphorylation by protein kinase A, protein kinase C, Ca^{++}-calmodulin-dependent protein kinase type II and tyrosine kinase (Gasic and Hollmann, 1992). Recent studies have demonstrated that activation of protein kinase C can induce potentiation of NMDA-induced current responses in rat spinal dorsal horn neurons (Gerber et al., 1989) in *Xenopus* oocytes injected with rat forebrain mRNA (Kelso et al., 1992), in trigeminal sensory nucleus (Chen and Huang, 1991) and in hippocampal neurons (Aniksztejn et al., 1991). In addition, protein kinase C also mediates the potentiation of NMDA responses by activation of metabotropic glutamate receptor in hippocampus (Aniksztejn et al., 1991) and by activation of μ opioid receptor in trigeminal sensory nucleus (Chen and Huang, 1991). Whereas the non-NMDA glutamate receptors seem to be affected by the activation of protein kinase C-dependent second messenger system in a less consistent manner (Gerber et al., 1989, Aniksztejn et al., 1991, Chen and Huang, 1991, Rusin et al., 1992, 1993, Cerne and Randic, 1993), they seem to be consistently upregulated by cyclic AMP-dependent second

messenger system. Recent studies show, that glutamate receptors gated by kainate in white perch retinal horizontal cells, mammalian hippocampal neurons, GluR6 glutamate receptor transiently expressed in mammalian cells, and GluR1/GluR3 glutamate receptors gated by AMPA expressed in Xenopus oocytes can be regulated by cyclic AMP-dependent protein phosphorylation (Liman et al., 1989, Chen and Huang, 1991, Greengard et al., 1991, Wang et al., 1991, Chavez-Noriega and Stevens, 1992, Keller et al., 1992, Raymond et al., 1993). Possible modulation of the NMDA receptors by cyclic AMP-dependent protein kinase remains less understood (Chen and Huang, 1991, Greengard et al., 1991, Wang et al., 1991, McVaugh and Waxham, 1992, Cerne et al., 1992). The modulation of the NMDA responses of DH neurons (Gerber et al., 1989, Rusin et al., 1992) and *Xenopus* oocytes injected with rat forebrain mRNA (McVaugh and Waxham, 1992) by cyclic AMP-dependent protein kinase has been suggested, and maintenance of NMDA-induced currents requires presence of ATP intracellularly (MacDonald et al., 1989). However, whole cell and single channel analysis revealed no obvious alterations of the NMDA channel properties in cultured hippocampal neurons (Greengard et al. 1991, Wang et al., 1991).

The presence of cyclic AMP-dependent second messenger and PKC-dependent second messenger system in the superficial dorsal horn, was demonstrated by high levels of forskolin and phorbol ester binding sites (Worley et al., 1986, Mochly-Rosen et al., 1987). The previous studies from our laboratory have demonstrated that phorbol esters, the activators of PKC, can modulate the synaptic transmission in the spinal dorsal horn through enhancement of presynaptic release of EAA and through increased

postsynaptic responsiveness of DH neurons to excitatory amino acids (Gerber et al., 1989c). The extraneuronal localization of PKC in spinal dorsal horn (Mochly-Rosen et al., 1987) and the possibility of activation of cyclic AMP-second messenger system by activation of PKC (Bell et al., 1985, Rozengourt et al., 1987) added to the complexity of these findings. The possible modulatory role of cyclic AMP-dependent second messenger system in modulation of excitatory amino acid mediated synaptic transmission was further supported by the fact that forskolin, an activator of adenylate-cyclase, enhances synaptic and excitatory amino acid responses in rat dorsal horn neurons (Gerber et al., 1989c, Rusin et al., 1992).

The existence of synaptic plasticity phenomena at the level of spinal dorsal horn (Randic et al., 1993) and the involvement of protein kinase systems in mediation of neuroplasticity phenomena in hippocampus, striatum and cerebellum, suggests the possible functional role of protein phosphorylation in the modulation of synaptic transmission in the spinal dorsal horn. Indeed, recent evidence suggests that cyclic AMP plays a role as a second messenger system in the hyperalgesia produced by agents acting on primary afferent terminals (Taiwo and Levine 1989 and 1991, Taiwo et al., 1990).

RATIONALE

The superficial spinal dorsal horn is an area where primary afferent fibers arising predominantly from skin, but also viscera and muscle, terminate and form first synaptic relay with dendrites of dorsal horn neurons. For this reason, the superficial spinal dorsal horn has been regarded as an important site for the initial processing of afferent signals directly related to the transmission and modulation of sensory information, including pain.

Anatomical and physiological studies have provided a detailed description of termination patterns of primary afferent fibers. Relatively well established is also the neurotransmitter role of glutamate in the mediation of fast excitatory synaptic transmission at the primary afferent synapses in the DH. Besides glutamate, primary afferent fibers contain more than ten neuropeptides that act through activation of second messengers and are thought to be involved in the modulation of primary afferent neurotransmission. Glutamate itself has also a modulatory role in primary afferent neurotransmission, since at least metabotropic glutamate receptor and NMDA subtype of ionotropic receptor are capable of activation of second messengers.

One of the best studied forms of neuroplasticity at central synapses is long term potentiation (LTP) at the hippocampal CA1 neurons. A similar form of synaptic plasticity has been recently described at the primary afferent synapse in the superficial dorsal horn. It has been recently demonstrated that induction of hippocampal LTP requires activity of

metabotropic glutamate receptor and that the LTP is modulated by the cyclic AMP-dependent second messenger system. Since the superficial dorsal horn contains high levels of cyclic AMP-dependent second messenger system and metabotropic glutamate receptor, it is likely that both systems play an important role in the modulation of the primary afferent synaptic transmission.

The present study attempted to estimate the possible modulation of excitatory amino activated-responses by the cyclic AMP-dependent second messenger system and by activation of metabotropic glutamate receptor. The particular interest of the study was to evaluate possible change in sensitivity of postsynaptic AMPA, KA and NMDA receptors of DH neurons by cyclic AMP and activation of mGluR. Modulation of ionotropic glutamate receptors has not been characterized in detail as yet, but may represent an important mechanism of regulation of synaptic efficacy at the primary afferent synapse.

We have utilized two different technical approaches. The first part of the study was done using the *in vitro* transverse spinal slice preparation. The standard sharp electrode voltage recordings were obtained from dorsal horn neurons. This preparation enabled us to record for prolonged periods of time from relatively intact dorsal horn neurons with preserved primary afferent-dorsal horn neuronal circuitry. It also allowed better visual access than the *in vivo* spinal cord preparation and fast application and removal of drugs. However, there are also limitations of the slice preparation studies such as diversity of neurons in the dorsal horn, difficulty to isolate single postsynaptic neuron and control its

membrane potential and internal milieu. Therefore in the second part of the study we utilized the preparation of acutely isolated DH neurons and whole-cell recordings of EAA-induced current responses. The latter approach allowed us isolation of postsynaptic sites, control of membrane potential and manipulateion of the intracellular environment by intracellular administration of large molecules, like catalytic subunit of cyclic AMP-dependent-protein kinase. The major disadvantages of this approach are the damage of neurons during the dissociation process and the alteration of normal intracellular environment.

In the present study we have presented evidence that the activation of the adenylate cyclase-cyclic AMP-dependent protein kinase system may be involved in the enhancement of primary afferent neurotransmission. In addition to increase of presynaptic release of neurotransmitters and depolarization of resting membrane potential, the cyclic AMP-dependent protein kinase system enhances sensitivity of postsynaptic AMPA, KA and NMDA receptors, probably through direct phosphorylation of receptor-channel complex or receptor channel complex-associated regulatory protein. The study further demonstrated that glutamate through its action at the metabotropic receptor enhances the postsynaptic responses of AMPA and NMDA glutamate receptor subtypes.

Our study suggests that in the rat spinal dorsal horn the adenylate cyclase-cyclic AMP-dependent second messenger system and the activation of metabotropic glutamate receptor may be involved in modulation and integration of sensory information including pain.

PAPER I. CYCLIC ADENOSINE 3'5'-MONOPHOSPHATE POTENTIATES EXCITATORY AMINO ACID AND SYNAPTIC RESPONSES OF RAT SPINAL DORSAL HORN NEURONS

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ABSTRACT

Intracellular recordings were made from rat dorsal horn neurons in the in vitro slice preparation to study the actions of cyclic adenosine 3',5'-monophosphate (cyclic AMP). In the presence of TTX, bath application of the membrane permeable analogue of cyclic AMP, 8-Br cyclic AMP (25-100μM) caused a small depolarization of the resting membrane potential accompanied by a variable change in membrane input resistance. In addition, 8-Br cyclic AMP caused a long-lasting increase in the spontaneous synaptic activity and the amplitude of presumed monosynaptic excitatory postsynaptic potentials evoked in the substantia gelatinosa neurons by orthodromic stimulation of a lumbar dorsal root. When the fast voltage-sensitive Na conductance was blocked by TTX, 8-Br cyclic AMP enhanced in a reversible manner, the depolarizing responses of a proportion of dorsal horn neurons to N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), quisqualic acid (QA) and kainic acid (KA). The effects of 8-Br cyclic AMP on the resting membrane potential and the NMDA response of dorsal horn neurons were mimicked by reducing phosphodiesterase activity with bath application of 3-isobutyl-1-methylxanthine, but not by cyclic AMP applied extracellularly. Moreover, we have found that intracellular application of a protein inhibitor of cyclic AMP-dependent protein kinase (PKI) into dorsal horn neurons prevents the 8-Br cyclic AMP-induced potentiation of the NMDA response of these cells. These results suggest that in the rat spinal dorsal horn the activation of the adenylate cyclase-cyclic AMP-dependent protein kinase

system may be involved in the enhancement of the sensitivity of postsynaptic excitatory amino acid (NMDA, AMPA, KA) receptors and modulation of primary afferent neurotransmission, including nociception.

INTRODUCTION

Neurotransmitter action at synapses can be regulated through activation of second messenger systems (31,33,47,50,63). One of the most studied second messengers is 3′,5′ cyclic adenosine monophosphate (cyclic AMP), that is produced by activation of adenylate cyclase and in turn activates cyclic AMP-dependent protein kinase (protein kinase A, PKA). Cyclic AMP binds to the regulatory subunit of PKA, releasing the catalytic subunit that catalyzes the phosphorylation of a wide variety of cytoplasmic and membrane proteins (7,26,63,73). Such phosphorylation can alter the functional properties of proteins and modulate various physiological processes, including neurotransmitter release (8,33,63), and voltage-gated (36,37,46,65) and ligand-gated channels (6,32,38,39,45,56,69,83). Recently, a new signalling mechanism for cyclic AMP has been discovered that is independent of kinase activation (52).

Glutamate, or a related amino acid, appears to be the major candidate for the fast and the slow excitatory neurotransmitter in the mammalian brain (13,54,86) and spinal cord (28-30,40-42,74,91). The functional diversity of glutamate is reflected by the presence of multiple receptors that can be classified into two groups: ionotropic and metabotropic (53,57,75,86). Ionotropic receptors contain integral cation-specific ion channels, whereas metabotropic receptors are coupled to phospholipase C via G-proteins. The ionotropic receptors are subdivided into two distinct subtypes: N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors activated by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)

and kainic acid (KA). Participation of both AMPA/kainate and NMDA receptors in the generation of the fast and the slow spinal EPSPs has been demonstrated (19-23,28-30,42,91). Recently AMPA/KA, NMDA and metabotropic receptors have been characterized by molecular biology cloning techniques (5,25,34,35, 44,51,58,61,72,87). The findings that the spinal dorsal horn contains high density of binding sites for [^{3}H]-forskolin (89), that forskolin, an adenylate cyclase activator and caffeine and theophylline inhibitors of phosphodiesterase enzymes, depolarize dorsal horn neurons and increase their responses to NMDA and QA (10,31,43), and that the glutamate and kainate responses of cultured hippocampal neurons are enhanced by cyclic AMP-dependent protein kinase (32,83), raised the possibility that cyclic AMP through the activation of protein kinase A may play a functional role in the excitatory synaptic transmission in the spinal dorsal horn by modulating signal transduction at various subclasses of excitatory amino acid (EAA) receptors. In this paper we report on evidence, that the elevation of intracellular concentration of cyclic AMP by membrane permeable analog, 8-Br cyclic AMP, and application of phosphodiesterase inhibitor, IBMX, depolarize rat spinal dorsal horn neurons, enhance primary afferent neurotransmission and the responses of dorsal horn neurons to specific EAA receptor ligands. In addition, specific protein kinase inhibitor (PKI), that binds with high affinity to active catalytic subunit of cyclic AMP-dependent protein kinase (16), prevented the 8-Br cyclic AMP-induced depolarization of the resting membrane potential and the potentiation of NMDA responses of dorsal horn neurons. Preliminary reports of some aspects of this work have been published (10,43).

MATERIALS AND METHODS

Transverse slices were obtained from Sprague-Dawley rats of both sexes (16-32 days old) by using a technique that has been described elsewhere (31,59,60). Briefly, after the animal was anesthetized with ether, a segment of the lumbosacral (L_4-S_1) spinal cord was dissected out and sectioned with a Vibratome to yield several transverse slices, 300-450 μm thick, with short (3-5 mm) dorsal rootlets. After the incubation for 1 h in oxygenated (95% O_2 + 5% CO_2) control solution (in mM; NaCl 124, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 10, pH 7.4 at 30 ± 1°C), a slice was transferred into a recording chamber, where it was submerged beneath an oxygenated superfusing medium (flow rate about 3 ml/min) containing lowered concentration of potassium ions (1.9 mM KCl). The use of a high-K^+ solution during cutting and incubation of the slices seemed to improve their viability as assessed electrophysiologically in the same preparation.

Conventional electrophysiological techniques were used for intracellular recording from dorsal horn neurons (laminae I-V), as described (59). Under visual control, a single fiber-filled glass microelectrode filled with 4 M potassium acetate (pH 7.2; DC impedance: 75-150 MΩ) was placed in the dorsal horn, and neurons impaled by oscillating the capacity compensation circuit of a high input impedance bridge amplifier (Neurodata; Axoclamp 2). Cells were activated synaptically by electrical stimulation of primary afferent fibers. A coaxial stainless steel stimulating electrode (o.d. of inner and outer electrodes being 25

and 200 μm, respectively; Frederick Haer Co.) positioned on a lumbar dorsal rootlet was used. Single (0.02-0.5 ms pulses, 1-25 V) stimuli to lumbar dorsal roots were used to elicit fast excitatory synaptic potential (EPSP). The synaptic responses were stored on diskettes of a digital oscilloscope (Nicolet, model 4092) until processed and printed out onto a digital plotter. A DC pen-recorder (Gould 220 or 2200S S) was used to record membrane potential and EAA responses continuously.

α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), quisqualic acid (QA; Cambridge Research Biochemicals, CRB), kainic acid (KA; CRB), and N-methyl-D-aspartic acid (NMDA; CRB, Tokris), were applied extracellularly by positive pressure ejection (1-5 kPa; Neuro Phore, Medical Instruments) from micropipettes (drug concentration: 10^{-4}-10^{-2} M for 10-200 ms) with tip diameters of 5-10 μm. Positioning of these micropipettes within 50-200 μm of the cell body reliably produced excitatory amino acid responses. Drugs applied via the superfusing medium were: 8-bromoadenosine 3'5'-monophosphate (8-Br cyclic AMP, sodium salt), 3,'5'cyclic adenosine-monophosphate (cyclic AMP) , 3-isobutyl-1-methyl-xanthine (IBMX), tetrodotoxin (TTX), all were obtained from Sigma (8-Br cyclic AMP, also from Aldrich). Stock solutions of 8-Br cyclic AMP, cyclic AMP and protein kinase inhibitor (PKI) were made in distilled water, and that of IBMX in dimethyl sulfoxide, and then frozen in aliquots to be used in single experiments. The aliquots were diluted in oxygenated Krebs solution prior to the bath administration. Protein kinase inhibitor (PKI, 45 μM/ml; activity 1 μg protein inhibits 4000 phosphorylating units of protein kinase A; Sigma)(16), in 4M K-acetate was applied intracellularly,

by allowing to leak from the electrode without any driving current. Most of results (Figs. 3-9) are expressed as percent of control response that was determined as the average of first three responses recorded prior to drug administration. They are reported individually or as mean ± S.E.M. For statistical analysis we used one way ANOVA and statistical significance between means was determined by Student-Newman-Keuls test: $^{*}P < 0.05$; $^{**}P < 0.01$.

RESULTS

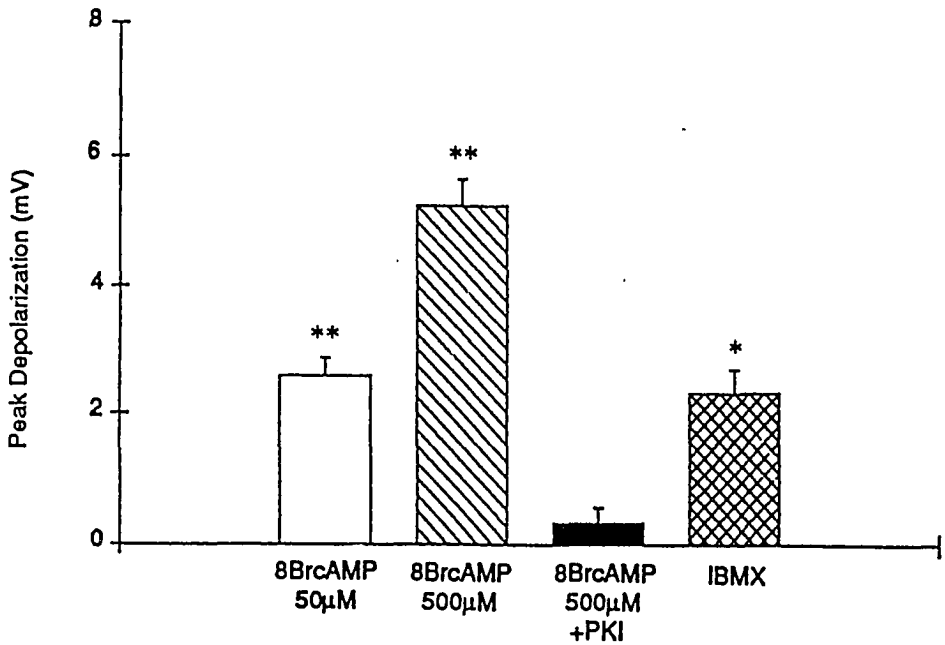
A total of 59 dorsal horn neurons in laminae I-V of the spinal dorsal horn was studied. Dorsal horn neurons exhibited mean resting potential and action potential amplitude of -73.0 ± 0.8 mV (m ± S.E.M.; range: -60 to -88 mV), and 81.4±2.9 mV (range: 56±102 mV), respectively.

Cyclic AMP reversibly depolarizes dorsal horn neurons

When synaptic transmission was blocked by tetrodotoxin (TTX, 5×10^{-7} M), bath application of the membrane permeable analogue of cyclic AMP, 8-Br cyclic AMP (2.5×10^{-5} - 10^{-3} M for 5-22 min) caused a small but long-lasting, dose-dependent (5×10^{-5} M cyclic AMP: 2.6 ± 0.3 mV, n=16; 5×10^{-4} M cyclic AMP: 5.3 ± 0.4 mV, n=12) depolarization of the membrane potential in 62% (n=42) of examined cells (Fig. 1). The onset of the effect was relatively slow (2.6 ± 0.5 min) and outlasted the time of bath application (by 11.2 ± 4.7 min). The membrane input resistance, as monitored by the amplitude of hyperpolarizing electrotonic potentials, showed either no apparent change or a small increase (average change about 10%) or decrease during 8-Br cyclic AMP-induced depolarization of the dorsal horn neurons. The slow time course of the depolarization made it possible to clamp manually the membrane potential during the response. The effects of 8-Br cyclic AMP on the membrane potential and input resistance of dorsal horn neurons were not mimicked by cyclic AMP applied extracellularly (n=2).

The depolarizing effect was present also in nine cells when the inhibitor of phosphodiesterase activity, 3-isobutyl-1-methyl xanthine

Fig. 1. Summarized results showing the depolarization of the membrane potential of rat spinal dorsal horn neurons caused by 8-Br cyclic AMP and IBMX, and the attenuation of the effect by PKI. In the presence of TTX ($5x10^{-7}M$), superfusion of spinal cord slices with 50μM (open bar, n=16) or 500 μM (hatched bar, n=12) 8-Br cyclic AMP for 5-22 min produced a dose-dependent depolarization of rat dorsal horn neurons. When microelectrodes containing PKI (100 mg/ml in 4M K-acetate, solid bar) were used, the 8-Br cyclic AMP-induced depolarization was markedly attenuated. Bath perfusion with a phosphodiesterase inhibitor, IBMX (10^{-4} - $2x10^{-3}M$; double-hatched bar) produced a depolarization that was similar in magnitude to the one evoked by 50μM 8-Br cyclic AMP. Statistical significance of results is marked by asterisk: $^{**}P<0.01$, $^{*}P<0.05$. Resting membrane potential (Vm) -72.6±0.8mV; 18-32-day old rats.


Peak Depolarization (mV)
8
6
4
2
0
**
**
*
8BrcAMP 50μM
8BrcAMP 500μM
8BrcAMP 500μM +PKI
IBMX

(IBMX; 10^{-4} - $2x10^{-3}$ M for 5-20 min) was bath-applied (Fig. 1).

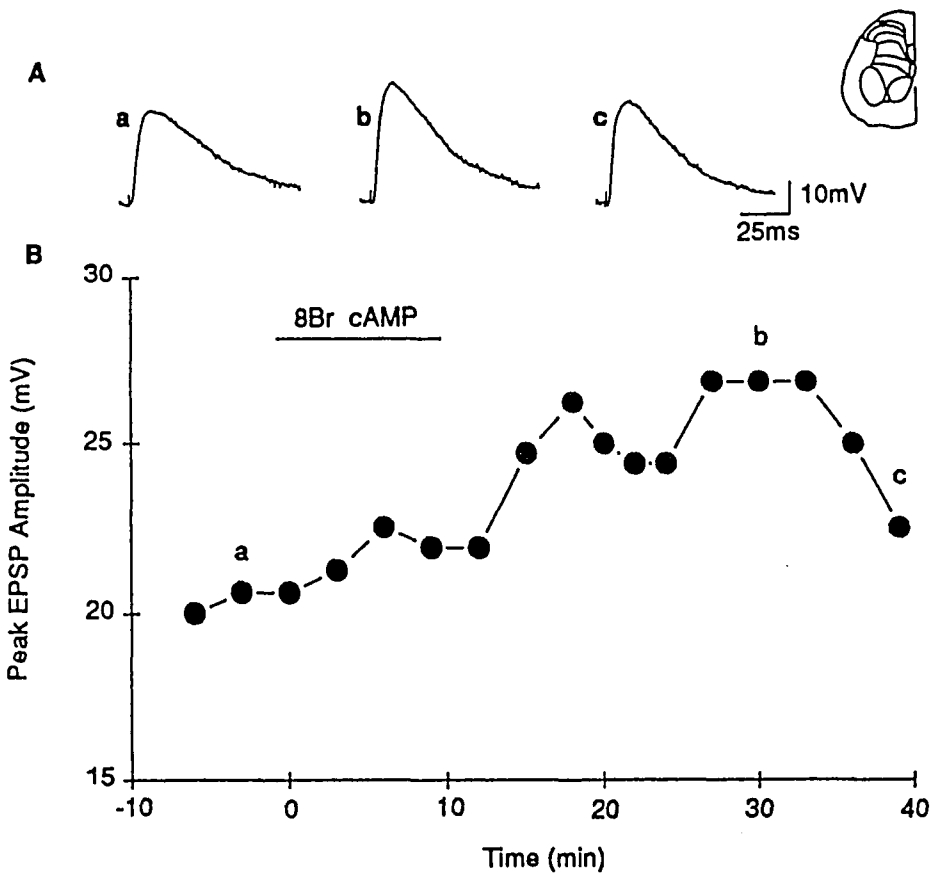
Next, we examined the possible involvement of cyclic AMP-dependent protein kinase in the membrane action of 8-Br-cyclic AMP by utilizing a highly specific protein inhibitor of cyclic AMP-dependent protein kinase (PKI; 16). When PKI (45 μM/ml) was intracellularly applied into neurons prior to 8-Br cyclic AMP application, the depolarizing effect of the 8-Br cyclic AMP was either substantially attenuated or completely blocked in five tested neurons (Fig. 1).

Potentiation of excitatory postsynaptic potentials by 8-Br cyclic AMP at primary afferent synapses with substantia gelatinosa neurons

When we used dorsal root electrical stimulation to evoke EPSPs in substantia gelatinosa neurons we obtained 10 of 14 neurons that followed the repetitive stimulation at 10 Hz, had constant delay after stimulus artifact and kept the same smooth shape throughout the experiment. On the basis of these criteria we presumed that these neurons are monosynaptically connected to primary afferent fibers. It has been earlier reported that a high proportion of primary afferent-evoked EPSPs recorded from the substantia gelatinosa neurons appear to be monosynaptic (90).

Bath application of 8-Br cyclic AMP ($2.5x10^{-5}$ - 10^{-4} M for 5-15 min) to a slice caused a prolonged, but reversible increase, in the peak amplitude of the fast presumed monosynaptic EPSPs (Fig. 2) evoked in the substantia gelatinosa neurons by electrical stimulation of a lumbar dorsal root. In several cells, the EPSP grew large enough to trigger action potentials.

Fig. 2. Potentiation of fast excitatory synaptic transmission at primary afferent synapses with neurons in substantia gelatinosa. A, an apparent monosynaptic EPSP evoked in a substantia gelatinosa neuron by single electrical stimuli (8.5V, 0.1ms) applied to a lumbar dorsal root prior to and after 8-Br cyclic AMP (50μM for 5 min) addition to the superfusing fluid. B, the graph shows the peak EPSP amplitude plotted as a function of time; letters (a-c) mark the individual responses illustrated above the graph (A). 8-Br-cyclic AMP was applied at time zero (arrow). Resting membrane potential (V_m): -85mV. 7-day-old rat. Inset in this and in the subsequent figures shows approximate location of a dorsal horn neuron determined by light microscopic inspection of the slices.


A
a
b
c
10mV
25ms
B
8Br cAMP
Peak EPSP Amplitude (mV)
30
25
20
15
-10
0
10
20
30
40
Time (min)

The onset of the action of 8-Br cyclic AMP varied from 3-9 min in differentsubstantia gelatinosa neurons, and the effect usually lasted between 5 and 20 min (Fig.2B). The potentiating effect was present in 9 of 10 tested cells, where it often occurred in the absence or after a minimal change in membrane potential and input resistance. The average enhancement in the EPSP amplitude amounted to 137.8±9.8% (mean ± S.E.M., n = 9).

With higher concentrations (10^{-4}-10^{-3}M for 5 min) of 8-Br cyclic AMP, however, a small decrease of the peak amplitude of EPSP was observed in all of 4 tested cells. With 10^{-3}M 8-Br cyclic AMP the depression varied between 40-62% of the control EPSP amplitude. The depressent effect was accompanied by a hyperpolarization of the membrane potential in 2 cells. In another two cells the EPSP was affected in a biphasic manner by addition of 8-Br cyclic AMP: during the presence of 8-Br cyclic AMP in the bath the EPSP was depressed and after wash-out of 8-Br cyclic AMP the EPSP was potentiated. One possibility that has not been excluded in these experiments is that the depression was caused by action of 8-Br cyclic AMP on adenosine receptors.

In more than half of tested cells (n=10), 8-Br cyclic AMP caused an increase in the frequency and amplitude of presumptive spontaneous EPSPs and occasionally evoked spike discharge. The response could have been generated by the firing of previously silent afferent fibers or spinal interneurons.

Cyclic AMP applied into a bath, the compound unable to penetrate the neuronal membrane, did not enhance the evoked EPSPs.

8-Br-cyclic AMP enhances the responsiveness of dorsal horn neurons to glutamate receptor agonists

The spinal dorsal horn, especially the substantia gelatinosa, contains high levels of binding sites for forskolin (89). Thus one possible mechanism that could account for the 8-Br cyclic AMP-induced increase of the synaptic strength in the substantia gelatinosa neurons is through the regulatory action of the cyclic AMP second messenger system in the signal transduction at various subclasses of excitatory amino acid receptors. Since most of examined dorsal horn neurons responded with depolarization of their membrane potential to glutamate and selective agonists of EAA receptors, the effects of cyclic AMP-active agents on the postsynaptic depolarizing responses of dorsal horn neurons to NMDA, AMPA, QA and KA were examined in the presence of TTX ($5x10^{-7}M$) in superfusing medium.

In experiments involving pressure application of NMDA we omitted Mg^{2+} ions from the bathing medium, since it is known that the NMDA ionophore is gated by Mg^{2+} ions in a voltage-dependent manner and that this block is partially relieved by removing extracellular Mg^{2+} (4,55,64). Glutamate receptor agonists (NMDA, AMPA, QA and KA) were applied at regular 2-3 min intervals by local pressure microinjection (10^{-4}-$10^{-2}M$ for 10-200ms, 1-5 kPa) in the vicinity (50-200 μm) of the recording sites. During testing of EAA responses, the membrane potential as a routine was manually clamped to its control value by injecting positive d.c. current through the microelectrode. A total of 48 neurons was tested with bath-applied 8-Br cyclic AMP ($2.5x10^{-5}$ - $2x10^{-3}M$); over 90% of the experiments were done using

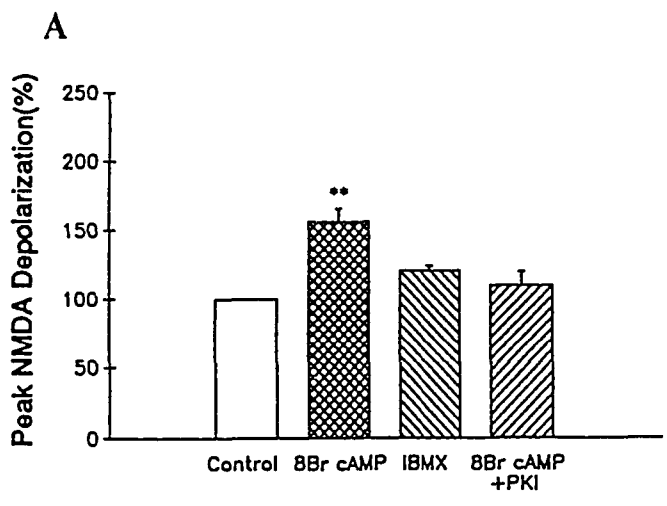
the concentrations of $5x10^{-5}M$ and $5x10^{-4}M$.

Enhancement of the NMDA responses by 8-Br cyclic AMP in rat dorsal horn neurons

A significant increase in the peak amplitude (to 172 ± 13.2% of control, n=9) and the half-decay time (to 156.3 ± 5.2%) of the depolarizing potential induced by NMDA was found after bath administration of 8-Br cyclic AMP in all examined cells (n=9), as shown in Figs. 3,4,6. After washing with a superfusing solution for 15-20 min, the NMDA response in some cells almost fully recovered (amplitude: to 107.3 ± 5.2%; duration: to 106.5 ± 3.7%). In general the onset of action of 8-Br cyclic AMP was slow (4.7±3.1 min) and outlasted the drug application on average by 16.1 ± 2.8 min.

One possibility that could account for the actions of 8-Br cyclic AMP on the rat dorsal horn neurons is that the compound might be inducing its effects by binding to extracellular adenosine receptors, rather than by promoting cyclic AMP-dependent intracellular processes. To examine this possibility, adenosine (1-50 μM) and cyclic AMP were bath-applied to dorsal horn cells. In contrast to 8-Br cyclic AMP, adenosine caused a dose-dependent hyperpolarization of the membrane potential accompanied by a decrease in membrane input resistance, in agreement with our previous report (43). For comparative reasons, 3 cells were tested with bath-applied 3',5'-cyclic AMP, the compound unable to penetrate the neuronal membrane. While 3',5'-cyclic AMP (100μM) either did not induce any change,

Fig. 3. Summarized results showing the enhancement of the NMDA response of dorsal horn neurons caused by 8-Br cyclic AMP or IBMX and blockade of the effect by PKI. In the presence of TTX ($5x10^{-7}M$) bath-applied 8-Br cAMP ($5x10^{-5}M$-$5x10^{-4}M$; n=9; double-hatched bar) or IBMX ($10^{-4}M$-$2x10^{-3}M$; n=4; hatched bar) produced increases in the peak amplitude (A) and in the half-decay-time (B) of the NMDA-induced depolarization. When the microelectrodes contained PKI (100 mg/ml) the enhancement of the NMDA responses of four dorsal horn neurons to 8-Br cAMP was prevented. Statistical significance in relation to the PKI-treated group is marked by asterisks. Vm, -72.6 ± 0.8 mV, 18-32-day-old rats.


A
Peak NMDA Depolarization(%)
250
200
150
100
50
0
**
Control
8Br cAMP
IBMX
8Br cAMP
+PKI

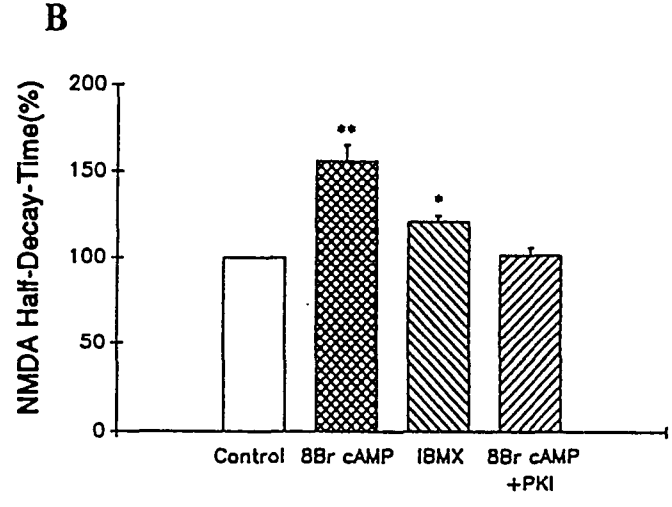
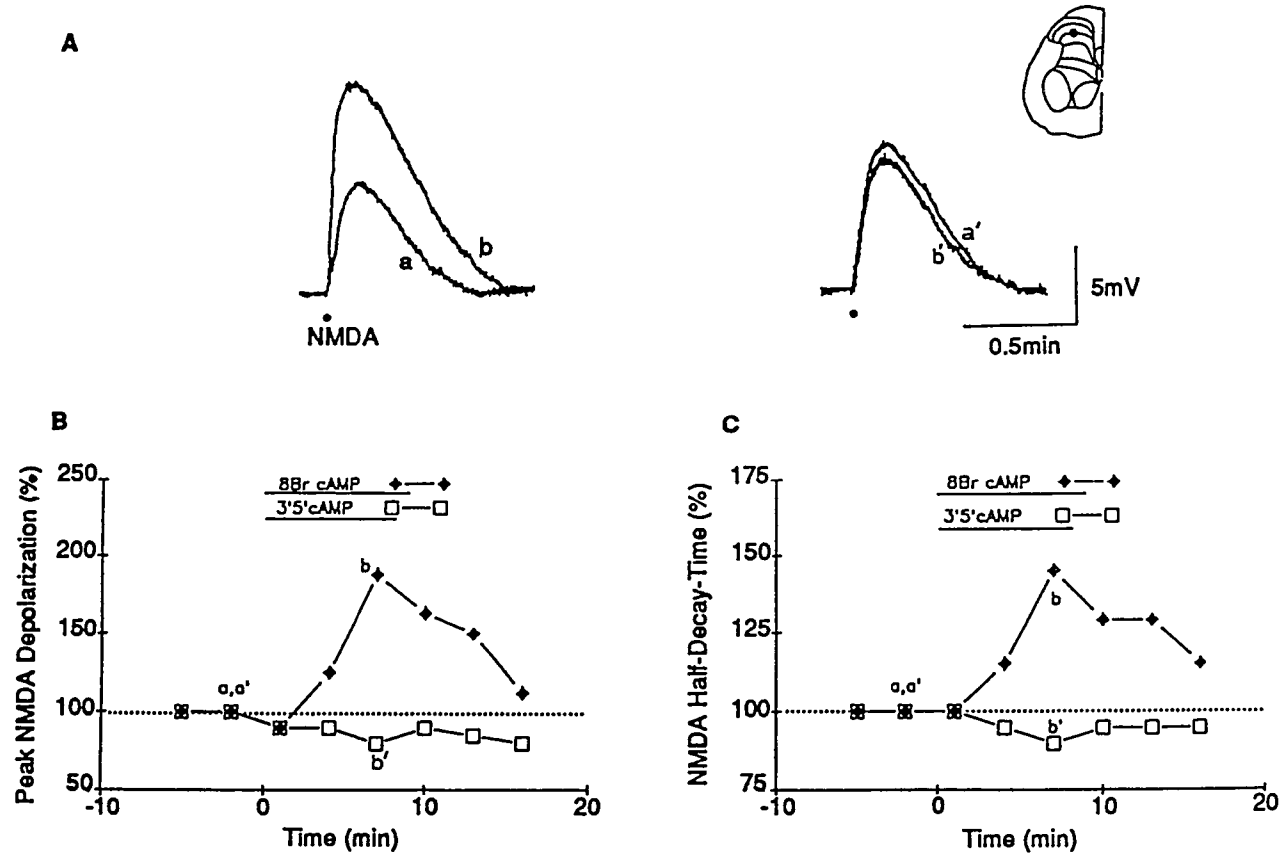

B
NMDA Half-Decay-Time(%)
200
150
100
50
0
**
*
Control
8Br cAMP
IBMX
8Br cAMP
+PKI

Fig. 4. 8-Br cyclic AMP, but not 3′,5′ cyclic AMP, increases sensitivity of a dorsal horn neuron to NMDA. In the presence of TTX (5×10^{-7}M), NMDA (10^{-2}M, 0.25s, 5kPa) was applied by positive pressure from micropipette with a tip diameter of about 5μm at 3 min intervals. Whereas the bath application of 3′,5′cAMP (5×10^{-4}M for 8 min) slightly depressed the depolarizing response to NMDA (Ab′), when the same neuron was superfused by 8-Br cAMP (5×10^{-4}M for 9 min) the depolarizing response was potentiated (Ab). Graphs show normalized values of the peak NMDA-induced depolarization (B) and the half-decay-time of the NMDA response (C), as a function of time. Small letters on the graphs denote the responses illustrated in A. Vm, -83mV, 32-day-old rat.


A
a
b
NMDA
a'
b'
5mV
0.5min
B
8Br cAMP
3'5'cAMP
Peak NMDA Depolarization (%)
250
200
150
100
50
a,a'
b
b'
-10
0
10
20
Time (min)
C
8Br cAMP
3'5'cAMP
NMDA Half-Decay-Time (%)
175
150
125
100
75
a,a'
b
b'
-10
0
10
20
Time (min)

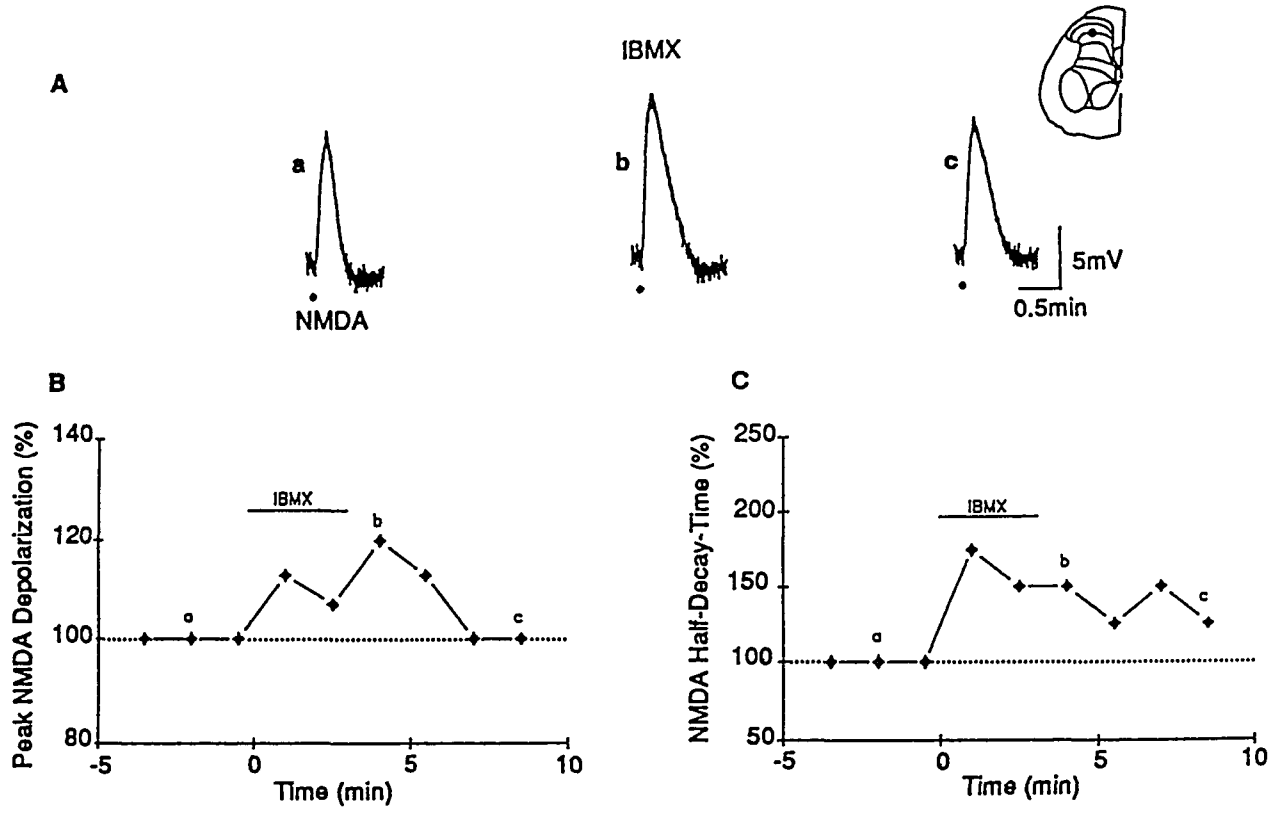
or caused even a small decline (Fig. 4B,C) in the sensitivity of dorsal horn neurons to NMDA, the NMDA response consistently increased after addition of 8-Br cyclic AMP to the same cells (Fig. 4A-C). Since these two agents, adenosine and cyclic AMP, did not mimic the actions of 8-Br cyclic AMP, and indeed had opposite effects on resting membrane potential and neuronal input resistance, it seems unlikely that 8-Br cyclic AMP produces its effects through an adenosine receptor.

Next, the effect of the endogenous cyclic AMP on the sensitivity of the postsynaptic membrane of dorsal horn neurons to NMDA was examined under conditions of reduced phosphodiesterase activity. If the 8-Br cyclic AMP-induced increase of the depolarizing response of dorsal horn neurons to NMDA is mediated through cyclic AMP then reducing the activity of this enzyme should potentiate the response to NMDA. To inhibit phosphodiesterase activity we used IBMX, an agent which has been shown to increase cyclic AMP levels in brain slice tissue (76). As illustrated in Figs. 3 and 5, bath-applied IBMX (10^{-4} - $2x10^{-3}$M) reversibly enhanced the peak amplitude (to 127.0 ± 8.7%) and the half-decay time (to 120.7 ± 3.5%) of the NMDA-induced depolarization in 4 cells.

The enhancement of the NMDA response by 8-Br cyclic AMP is attenuated by a specific inhibitor of cyclic AMP-dependent protein kinase (PKI)

Cyclic AMP-dependent protein kinase (protein kinase A, PKA) has been postulated to mediate most, if not all, of the effects of cyclic AMP in nerve cells (63). Therefore, we next examined whether the intracellular

Fig. 5. Phosphodiesterase inhibitor IBMX increases the depolarizing response of a dorsal horn neuron to NMDA. A, in the presence of TTX ($5x10^{-7}$M), bath applied IBMX ($5x10^{-4}$M, for 3.5 min) enhanced the depolarization produced by pressure application of NMDA (10^{-2}M, 0.16s, 5kPa). Graphs show the time course of changes in the peak NMDA-induced depolarization (B) and the half-decay-time (C). Vm, -70mV, 19-day-old rat.


A
IBMX
a
b
c
5mV
0.5min
NMDA
B
C
Peak NMDA Depolarization (%)
140
120
100
80
IBMX
a
b
c
-5
0
5
10
Time (min)
NMDA Half-Decay-Time (%)
250
200
150
100
50
IBMX
a
b
c
-5
0
5
10
Time (min)

injection of a highly specific protein inhibitor of PKA, (PKI, 16) could block the effect of 8-Br cyclic AMP in order to establish that PKA, possibly through protein phosphorylation, was mediating the actions of 8-Br cyclic AMP in the rat dorsal horn neurons. When PKI (45 μM/ml) was applied intracellularly for 40-65 min prior to 8-Br cyclic AMP ($5x10^{-4}$M) administration, the increase in the NMDA-induced depolarization usually seen after 8-Br cyclic AMP, was either markedly reduced or completely abolished in 4 cells (Fig. 3 and Fig. 6A, right traces). The latter result indicates that activation of PKA in the neuron being recorded from is required for the enhancement of the NMDA response. This finding however, did not rule out the possible contribution of presynaptic effects.

8-Br cyclic AMP enhances the depolarizing responses of dorsal horn neurons to AMPA, QA, and KA

In addition to the findings with NMDA, the postsynaptic depolarizations induced by local pressure microinjection of AMPA (Fig 7), QA (Fig. 8) and KA (Fig. 9) were, in the presence of TTX, also affected after bath application of 8-Br cyclic AMP in a proportion of rat dorsal horn neurons. 8-Br cyclic AMP, bath-applied in concentrations of 50-500 μM for 8-16 min increased the peak amplitude of the AMPA-induced depolarization (to 142.5 ± 12%) in 5 of 8 tested cells, but consistently increased the duration (to 143.7 ± 8.9%, n=8) of the AMPA response in all cells examined. The decrease in the amplitude of the AMPA response was present in 2 cells and one cell was not affected. An example of the

Fig. 6. PKI inhibits the enhancing effect of 8-Br cAMP on the NMDA-induced depolarization. In the same area of a slice, intracellular recordings were made from two different dorsal horn neurons in the presence of TTX ($5x10^{-7}M$). Whereas in the neuron impaled with 4M K-acetate-filled microelectrode, Vm, -78mV), bath-applied 8-Br cyclic AMP ($5x10^{-4}M$ for 9 min) potentiated (Ab) the depolarizing response to NMDA ($10^{-2}M$, 0.1s, 5kPa), in the second neuron impaled by 4M K-acetate + PKI (45μM/ml) - filled microelectrode (Vm, -80mV), 8-Br cyclic AMP ($5x10^{-4}M$ for 11 min) was without simmilar effect (Ab'). Graphs show normalized values of the peak NMDA-induced depolarization (B) and the half decay time (C). 25-day-old rat.

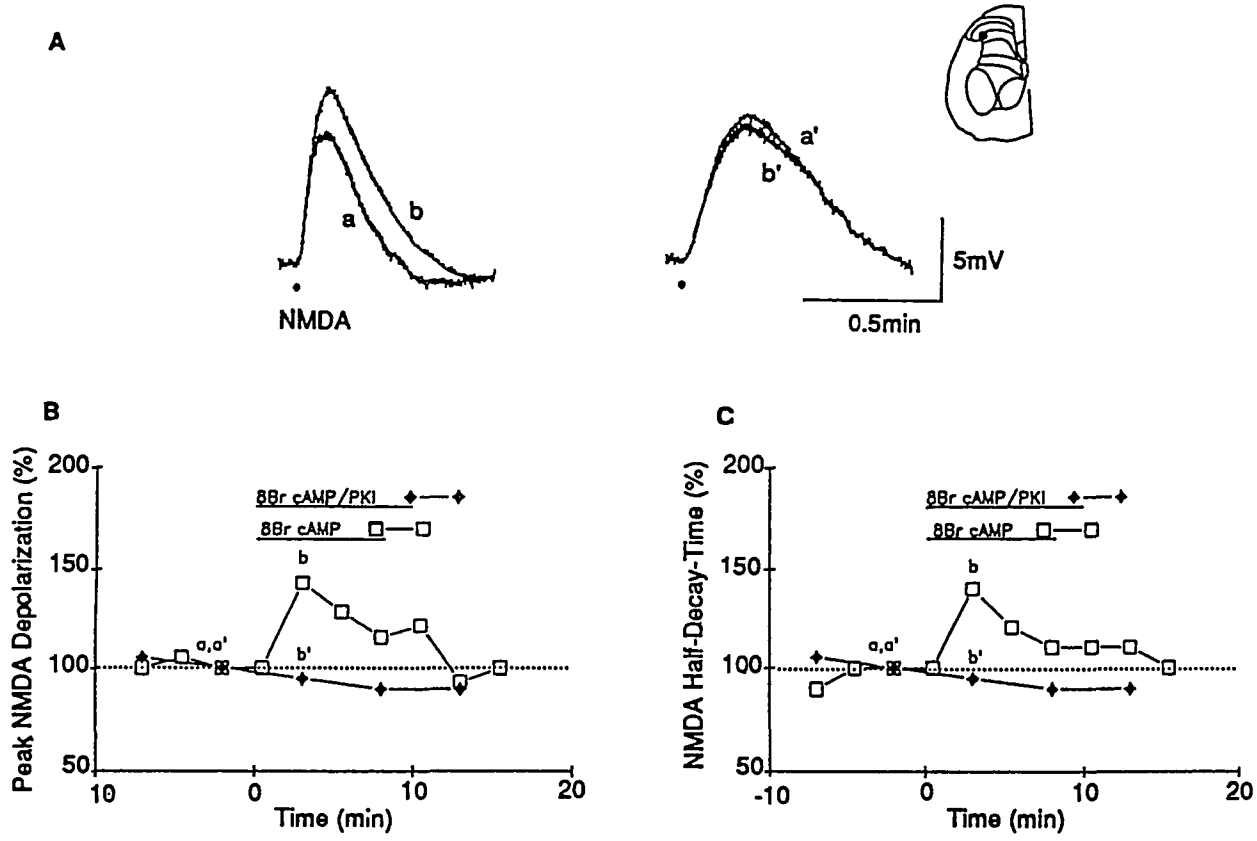
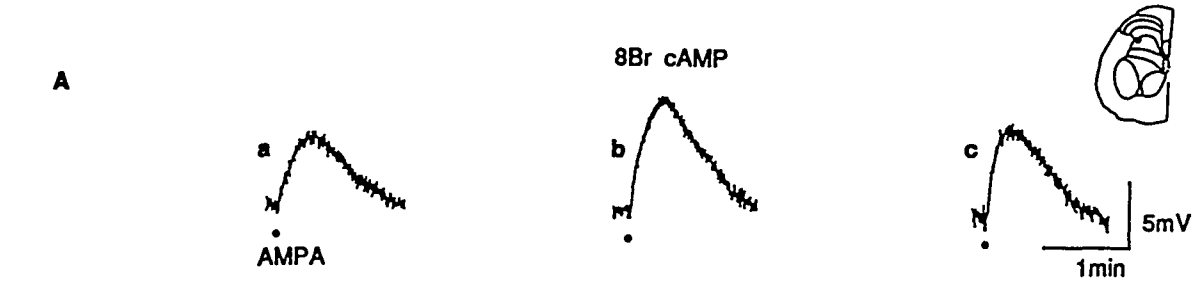
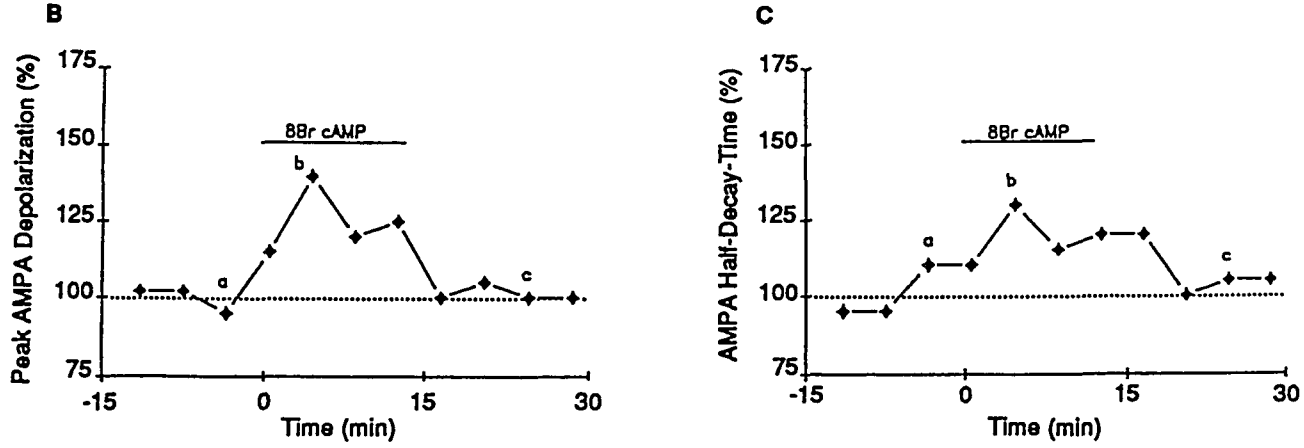

A
a
b
NMDA
a'
b'
5mV
0.5min
B
Peak NMDA Depolarization (%)
200
150
100
50
8Br cAMP/PKI
8Br cAMP
a,a'
b
b'
10
0
10
20
Time (min)
C
NMDA Half-Decay-Time (%)
200
150
100
50
8Br cAMP/PKI
8Br cAMP
a,a'
b
b'
-10
0
10
20
Time (min)

Fig. 7. 8-Br cyclic AMP increases sensitivity of a dorsal horn neuron to AMPA. In the presence of TTX ($5x10^{-7}M$), bath applied 8-Br cAMP ($5x10^{-5}M$ for 11 min) enhances the depolarizing response (Ab) of a dorsal horn neuron (inset) to AMPA applied by pressure ejection ($5x10^{-4}M$, 60ms, 0.9 kPa). Graphs show the normalized values of the AMPA-induced peak depolarization (B) and half-time-duration (C) of the AMPA responses. Vm, -78mV, 26-day-old rats.


A
8Br cAMP
a
b
c
AMPA
5mV
1min


B
Peak AMPA Depolarization (%)
175
150
125
100
75
8Br cAMP
a
b
c
-15
0
15
30
Time (min)

C
AMPA Half-Decay-Time (%)
175
150
125
100
75
8Br cAMP
a
b
c
-15
0
15
30
Time (min)

Fig. 8. 8-Br cyclic AMP increases sensitivity of a dorsal horn neuron to QA. In the presence of TTX ($5x10^{-7}M$), bath-applied 8-Br cyclic AMP ($5x10^{-4}M$ for 7 min) augmented the depolarizing response (Ab) of a dorsal horn neuron (inset) to QA applied by pressure ejection ($2.5x10^{-4}M$, 40ms, 2.7 kPa). Graphs show the normalized values of the QA-induced depolarization (B) and half-time duration (C) of the QA-response. Vm, -74mV, 21-day-old rat.

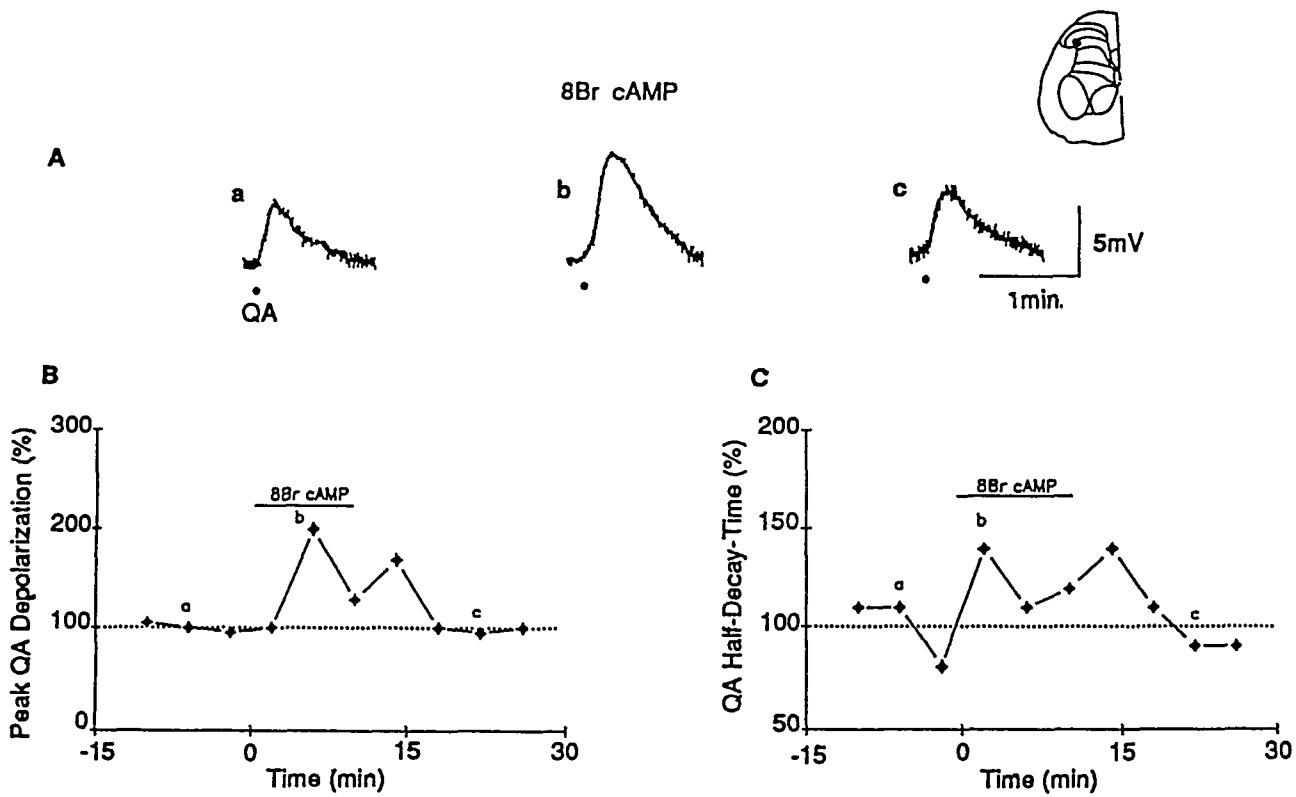
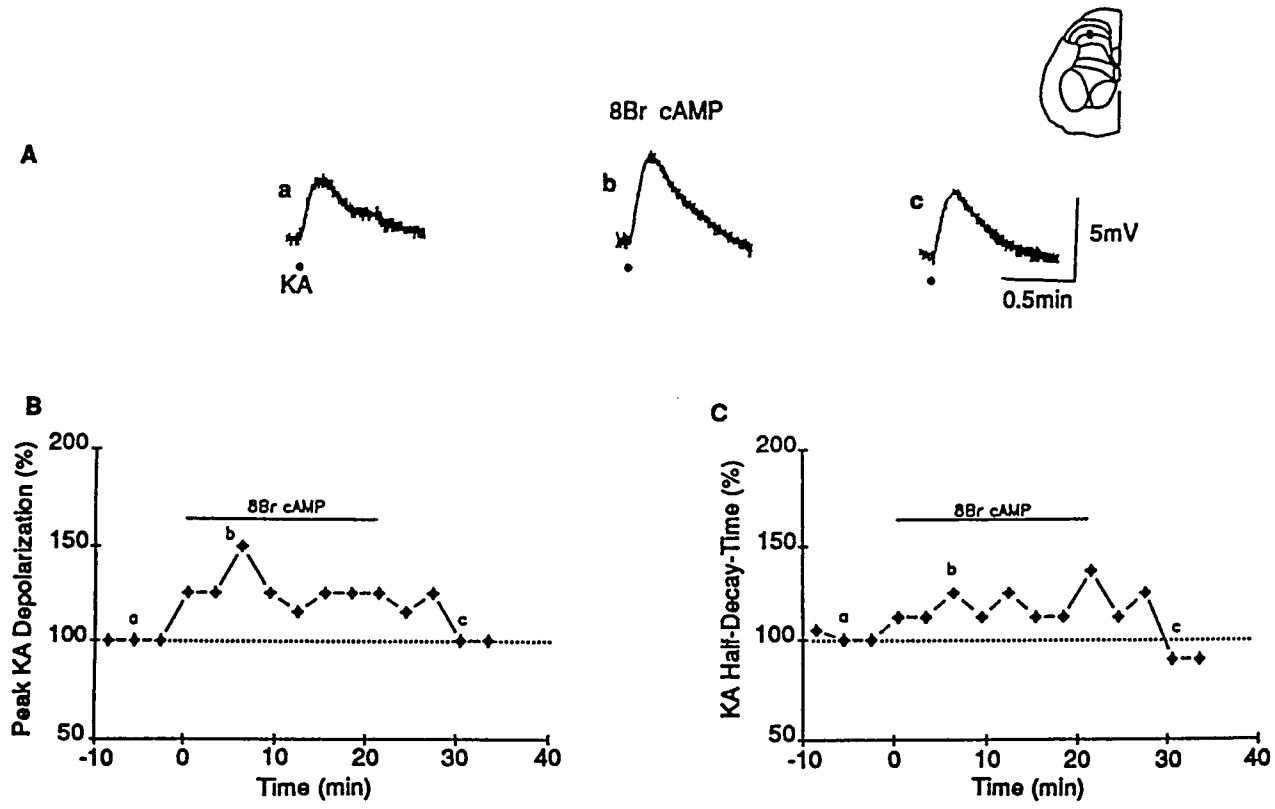

A
8Br cAMP
a
b
c
QA
5mV
1min.
B
Peak QA Depolarization (%)
300
200
100
0
8Br cAMP
-15
0
15
30
Time (min)
C
QA Half-Decay-Time (%)
200
150
100
50
8Br cAMP
-15
0
15
30
Time (min)

Fig. 9. 8-Br cyclic AMP increases sensitivity of a dorsal horn neuron to KA. In the presence of TTX ($5x10^{-7}$M), bath-applied 8-Br cyclic AMP ($5x10^{-4}$M for 22 min) enhanced the depolarizing response (Ab) of a dorsal horn neuron (inset) to KA applied by pressure ejection (10^{-2}M, 0.15s, 0.9kPa). Graphs show the normalized values of the KA-induced depolarization (B) and half-time duration (C) of the KA-response. Vm, -72mV, 25-day-old rat.


A
8Br cAMP
a
b
c
KA
5mV
0.5min
B
Peak KA Depolarization (%)
200
150
100
50
8Br cAMP
-10
0
10
20
30
40
Time (min)
C
KA Half-Decay-Time (%)
200
150
100
50
8Br cAMP
-10
0
10
20
30
40
Time (min)

enhancement of the AMPA response by 8-Br cyclic AMP is shown in Fig. 7.

8-Br cyclic AMP reversibly increased the peak amplitude (to 120.9 ± 6.8%) and half-decay time (to 164.2 ± 11.7%) of the QA-induced depolarization in 8 of 15 dorsal horn neurons examined (Fig. 8). However, in 2 cells a reduction in the QA response was observed.

The KA-induced depolarization was increased in the peak amplitude in 4 of 9 neurons (to 142.7 ± 7.3%) and the half decay-time (to 139.1 ± 4.8%) in 7 neurons (Fig. 9) after application of 8-Br cyclic AMP (50-500 μM for 5-20 mins).

DISCUSSION

The results presented in this paper indicate that the intracellular second messenger, the adenylate cyclase-cyclic AMP-dependent protein kinase system, potentiates the fast excitatory synaptic potentials at primary afferent synapses with substantia gelatinosa neurons. In addition, activation of this system modifies the resting membrane properties of dorsal horn neurons, enhances their responsiveness to excitatory amino acids and increases the probability of neurotransmitter release, as suggested by an increase in the frequency of spontaneous postsynaptic potentials.

Cyclic AMP (25-100μM), applied in the form of the membrane permeable analogue, 8-Br cyclic AMP, causes a small depolarization of the resting membrane potential. This finding is consistent with the results obtained in the rat hippocampal CA1 pyramidal neurons (49) and the locus coeruleus neurons (84), but it differs from the early studies in the hippocampus showing that cyclic AMP, or dibutyryl cAMP, caused inhibition of spontaneous activity and hyperpolarization of the CA1 cell membrane potential (78).

Cyclic AMP enhances the sensitivity of dorsal horn neurons to exogenous excitatory amino acids

When synaptic transmission was blocked by TTX, 8-Br cyclic AMP enhanced, in a reversible manner, the depolarizing responses of most dorsal

horn neurons to NMDA, and in a proportion of examined cells to AMPA, QA, and KA. In addition we found that the exposure of slices to forskolin (10), an activator of the catalytic subunit of adenylate cyclase (77) or IBMX, a phosphodiesterase inhibitor, produces effects similar to the 8-Br cyclic AMP on the responses of dorsal horn cells to NMDA.

Our finding of heterogeneity in the population of responses of dorsal horn neurons to exogenous excitatory amino acids may be related to heterogeneous population of neurons existing in the rat spinal dorsal horn, including the outer layers.

Although in our experiments the elevation of the intracellular cyclic AMP concentration enhanced the sensitivity of EAA receptors in a proportion of the rat spinal dorsal horn neurons, the precise molecular mechanisms involved and the identity of endogenous substance(s) participating in the effect is presently unknown. To test the hypothesis that activation of PKA in the dorsal horn neuron being recorded from is required for the enhancement of NMDA responses, a highly specific inhibitor of PKA (16) was included into microelectrode solution and allowed to dialyze into cells. The PKI (in the range of 1-5 μM when tested at or below Km values of appropriate protein substrates) inhibits catalytic activity of PKA by interacting specifically with free catalytic subunits dissociated from the holoenzyme in response to stimulation by cyclic AMP (2,3,16). In the presence of PKI (45 μM/ml), as our data show, the depolarization of the resting membrane potential (Fig. 1) and the enhancement of the NMDA responses of rat dorsal horn neurons caused by 8-Br cyclic AMP, were markedly attenuated (Figs. 3 and 6). These data suggest that PKA, possibly

through protein phosphorylation, may be involved in the modulation of the NMDA receptor function. In dorsal root ganglia (DRG) neurons (14) some invertebrate neurons (1,9,18,65), and rat locus coeruleus neurons (85), the electrophysiological effects of cyclic AMP-active agents have been reported to be inhibited by the same specific inhibitor of PKA enzyme as was employed in this study.

Two recent reports have suggested a role for the cyclic AMP-dependent protein kinase system in the regulation of the function of AMPA/KA receptors and fast excitatory synaptic transmission (32,83). In these electrophysiological studies the currents induced by activation of glutamate and AMPA/KA receptors were potentiated by agents that activate adenylate cyclase (forskolin) and that specifically modulated cyclic AMP-dependent protein kinase activity. Thus, the current responses were potentiated by intracellular application of the catalytic subunit of protein kinase A and depressed by a competitive inhibitor of this enzyme. Single channel analysis revealed that a cyclic AMP-dependent PKA increases the opening frequency and the mean open time of the non-NMDA-type glutamate receptor channels. These results suggested either that the non-NMDA channels themselves are phosphorylated directly or that their function is regulated indirectly through phosphorylation of membrane proteins.

Although modulation of the NMDA responses of rat dorsal horn neurons by protein kinase C (15,31) and protein kinase A (31) has been suggested by previous and the present studies, single channel analysis revealed no obvious alterations of the NMDA channel properties in cultured hippocampal neurons (32). However, in the whole-cell voltage-clamped rat dorsal horn

neurons we recently observed the enhancement of the NMDA-induced current responses upon intracellular application of the catalytic subunit of protein kinase A (unpublished observations). The latter result contrasts with the result of Greengard et al. (32), who concluded that modulation of NMDA channels by PKA is absent or at least less prominent than modulation of non-NMDA receptor channels. It is not clear why these differences exist but they could be due to differences in the preparation used and multiplicity of NMDA receptors.

Cyclic AMP enhances synaptic responses of dorsal horn neurons

The results presented in this paper indicate that the adenylate cyclase-cyclic AMP-dependent protein kinase system, potentiates the fast excitatory synaptic transmission in the rat spinal dorsal horn. There are several possible sites of action for 8-Br cyclic AMP in the slice preparation. It can enhance the release of neurotransmitter(s) from presynaptic terminals, it can enhance the responsiveness of postsynaptic neurons to released neurotransmitter(s), or it can increase the interneuronal activity in the dorsal horn. In the absence of significant changes in resting membrane potential and neuronal input resistance, the increased synaptic efficacy, as manifested by the increase in the amplitude of the evoked EPSP in the substantia gelatinosa neurons following administration of 8-Br cyclic AMP, could be due at least in part to increased probability of release of vesicles presynaptically (8,32). Previous studies suggested that cyclic AMP enhances transmitter release

form DRG terminals (19,79). Consistent with this possibility is our finding of an increase in the frequency of spontaneous EPSPs after the application of 8-Br cyclic AMP. However, the increase in the synaptic strength could also be due to a postsynaptic effect since we have shown that cyclic AMP-active agents modify the postsynaptic responses of a proportion of dorsal horn neurons to selective agonists of EAA receptors, including NMDA. However, use of intracellular voltage recordings from a relatively intact slice preparation and the fact that the membrane potential is the final common output of a number of pre- and postsynaptic processes makes it difficult task to assign conclusively a locus or mechanism to the effects produced by bath application of cAMP analogues.

In addition, there is a problem relating the changes in EAA responses to changes in responses to afferent stimulation. As is the case in the hippocampus, there may be extrajunctional EAA receptors on spinal neurons and these may be regulated differently from the receptors that participate in normal synaptic transmission. Thus, while activation of PKA may indeed enhance neuronal sensitivity to exogenous NMDA, additional or different mechanisms may account for the increase observed in the monosynaptic EPSP. In order to resolve this issue, it will be necessary in a future work to isolate the NMDA component of the excitatory postsynaptic current.

Physiological consequences of cyclic AMP actions for the slow excitatory synaptic transmission

We have recently demonstrated that the slow excitatory synaptic response (slow EPSP) evoked in the rat spinal dorsal horn neurons by repetitive stimulation of primary afferent fibers has two depolarizing components: an initial transient component that appears to require the activation of NMDA and AMPA/KA receptors (29,30) and a late, longer-lasting, possibly peptidergic component (70,71). Although the molecular mechanism of the slow EPSP in the rat dorsal horn is presently not completely understood, at least two ionic mechanisms are thought to be involved in the generation of the slow EPSP: a conductance decrease of a voltage-dependent K^+ current (11,88) and a conductance increase possibly to Na^+ and/or Ca^{2+} ions (11). In addition, the increase in Ca^{2+} influx and/or the change in intracellular free Ca^{2+} concentration are important during the late phase of the slow depolarizing response (11). The slow membrane depolarization, the long-lasting enhancement in excitability and the high temperature sensitivity of the slow EPSP (30) suggest that synthesis of a second messenger might be involved in a signal transduction during the slow EPSP. Consistent with this possibility are the findings that glutamate receptors can regulate the production of cyclic nucleotides in central neurons (27) and that NMDA receptor activation increases cyclic AMP levels and voltage-gated Ca^{2+} channel activity in area CA1 of hippocampus (17).

Although second messenger function of cyclic AMP in vertebrate neurons has long been suggested (33) the observed electrophysiological correlates

were predominantly inhibitory in nature (24,48,68). However, Palmer et al. (66,67), working in guinea pig myenteric neurons, provided evidence that cyclic AMP may function as a mediator of a slow excitatory synaptic transmission and transduction of some of peptidergic signals. Moreover, the experimental procedures that increase intracellular cAMP level simulate and potentiate the electrically evoked slow EPSP in both myenteric and dorsal horn neurons (43,62,66,67,92). In this context it is perhaps relevant that the mRNA for SP receptor, the receptor which appears to be functionally involved in the slow excitatory synaptic transmission in the rat spinal dorsal horn (29,70,71,82), is significantly increased by the agents that increase intracellular cyclic AMP levels.

In conclusion, our results suggest that in the rat spinal dorsal horn the activation of the adenylate cyclase-cyclic AMP-dependent protein kinase system may be involved in the regulation of the sensitivity of postsynaptic excitatory amino acid receptors and primary afferent neurotransmission, including nociception. Recent evidence has suggested that cyclic AMP plays a role as a second messenger system in the decrease of nociceptive threshold (or hyperalgesia) produced by the agents acting on primary afferent terminals (80,81).

Acknowledgments

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PAPER II. ENHANCEMENT OF THE N-METHYL-D-ASPARTATE RESPONSE IN SPINAL DORSAL HORN NEURONS BY cyclic AMP-DEPENDENT PROTEIN KINASE

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SUMMARY

Glutamate-gated ion channels mediate excitatory synaptic transmission in the central nervous system and are involved in synaptic plasticity, neuronal development and excitotoxicity (5,24). These ionotropic glutamate receptors were classified according to their preferred agonists as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), KA (kainate), and NMDA (N-methyl-D-aspartate) receptors (27). The present study of NMDA receptor channels expressed in acutely isolated spinal dorsal horn (DH) neurons of young rat reveals that these channels are subject to modulation through the adenylate cyclase cascade. Whole-cell voltage-clamp recording mode was used to examine the effect of adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase (PKA) on the responses of DH neurons to NMDA. Whole-cell current response to NMDA was enhanced by 8 Br-cyclic AMP, a membrane permeant analog of cyclic AMP or by intracellular application of cyclic AMP or catalytic subunit of PKA. Our results indicate that NMDA receptors are modulated by PKA and that this modulation is potentially important mechanism to control excitability of spinal DH neurons and the efficacy of synaptic transmission.

INTRODUCTION

There is evidence that second messenger systems can regulate neuronal excitability by phosphorylation of voltage-gated (10,11) and ligand-gated channels (13,16). Protein phosphorylation of glutamate receptors by protein kinase C (PKC) and cyclic AMP-dependent protein kinase (PKA) has been suggested to regulate their function (1,4,5,6,7,12,26) and be involved in some forms of synaptic plasticity such as long-term potentiation and long-term depression (24).

Whereas glutamate receptors gated by kainate in white perch retinal horizontal cells, mammalian hippocampal neurons, and GluR6 glutamate receptor transiently expressed in mammalian cells can be regulated by cyclic AMP-dependent protein phosphorylation (3,7,14,22,26), possible modulation of NMDA receptors remains less understood (1,4,7,17,20,26). Although the modulation of the NMDA responses of rat DH neurons by both protein kinase C (4,6) and protein kinase A (1,6) has been suggested, whole cell (26) and single-channel analysis (7) revealed no obvious alterations of the NMDA channel properties in cultured hippocampal neurons. However, the findings that the spinal dorsal horn contains high density of binding sites for forskolin (28) and that cyclic AMP potentiates the fast excitatory synaptic potentials at primary afferent synapses with substantia gelatinosa neurons and also enhances the responses of both non-NMDA and NMDA receptors of DH neurons in the spinal slice preparation (1,2) suggest that cyclic AMP-dependent second messenger system may have a functional role by modulating signal transduction at both subclasses of

glutamate receptor. Here we report that currents induced by activation of the NMDA receptor were potentiated by 8-Br cyclic AMP, the membrane permeable analogue of cyclic AMP, and by intracellular application of the catalytic subunit of PKA or cyclic AMP. Part of these results has been presented elsewhere (2,20).

METHODS

Single spinal DH neurons in the Rexed's laminae I-IV were isolated acutely from 9- to 16-day-old Sprague-Dawley rats by the method previously described (19). The isolated cells remain in a good condition for ≤ 8 h, exhibiting a variety of voltage-dependent ionic currents and having good responses to EAAs and peptides (19). The whole-cell voltage clamp technique (8) was used to record membrane currents of DH neurons at room temperature (20-23°C). Currents elicited by EAA and holding voltages were monitored continuously with a List L/M-EPC7 patch-clamp amplifier and recorded on a Gould-Brush pen recorder. In addition, currents were filtered at 2kHz, sampled at 2 kHz, stored on computer, and both acquired and analyzed using PCLAMP software (Axon Instruments). The solution perfusing the outside of the cell had the following composition (mM): NaCl 150, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ zero, HEPES 10, D-glucose 10, NaOH to adjust pH to 7.4, bovine serum albumin 0.1 mg/ml, and tetrodotoxin ($5x10^{-7}$M). In a majority of experiments, nominally glycine-free solutions, which are likely to contain ≤ 20nM glycine (the deionized water used to prepare recording and testing solutions contains a background glycine concentration of about 20 nM, as determined by high performance liquid chromatography), were used. However, in some of the experiments, the extracellular solution contained 10-100 nM glycine to increase the NMDA response (9). Electrodes were filled with one of two internal solutions containing (in mM): CsCl 140 (or 120 K-aspartate and 20 KCl), NaCl 10, $MgCl_2$ 1, $CaCl_2$ 0, HEPES 10, EGTA 1 and TRIS base for pH 7.2. To reduce wash-out of NMDA responses 3 MgATP,

0.1 GTP and 0.1 leupeptine or 6mM MgATP were added to internal solution. Membrane potential was clamped at -60 mV, where no holding current (leak current) was usually observed. NMDA (10^{-4}M; sodium salt, Tocris, Research Biochemicals Incorporated, RBI), 8-bromoadenosine 3'5'monophosphate (10^{-6}-10^{-4}M, 8-Br cyclic AMP sodium salt, Sigma), 3-isobutyl-1-methyl-xanthine ($5x10^{-4}$M, IBMX, Sigma), and tetrodotoxin ($5x10^{-7}$M, TTX, Sigma) were dissolved in the HEPES-buffered solution and applied to the recorded cells by a fast pressure system (19). NMDA was applied at low frequency (minimum rate of once every 2.5 min) to minimize desensitization. MgATP (6mM, Sigma), cyclic AMP (40-200μM, Sigma) and cPKA (20μg/ml, obtained as a generous gift from Dr. A.C. Nairn, Rockefeller University, or purchased from PROMEGA) were freshly prepared before each experiment and applied intracellularly using double-filled micropipettes, where the tip of the pipette was filled first with internal solution and shank with the internal solution containing MgATP or MgATP + cPKA or MgATP + heat inactivated cPKA (we boiled an aliquot of cPKA for 15 min to denature the enzyme) or cyclic AMP alone. cyclic AMP was also internally applied through a plastic tube inserted in the patch pipette (4).

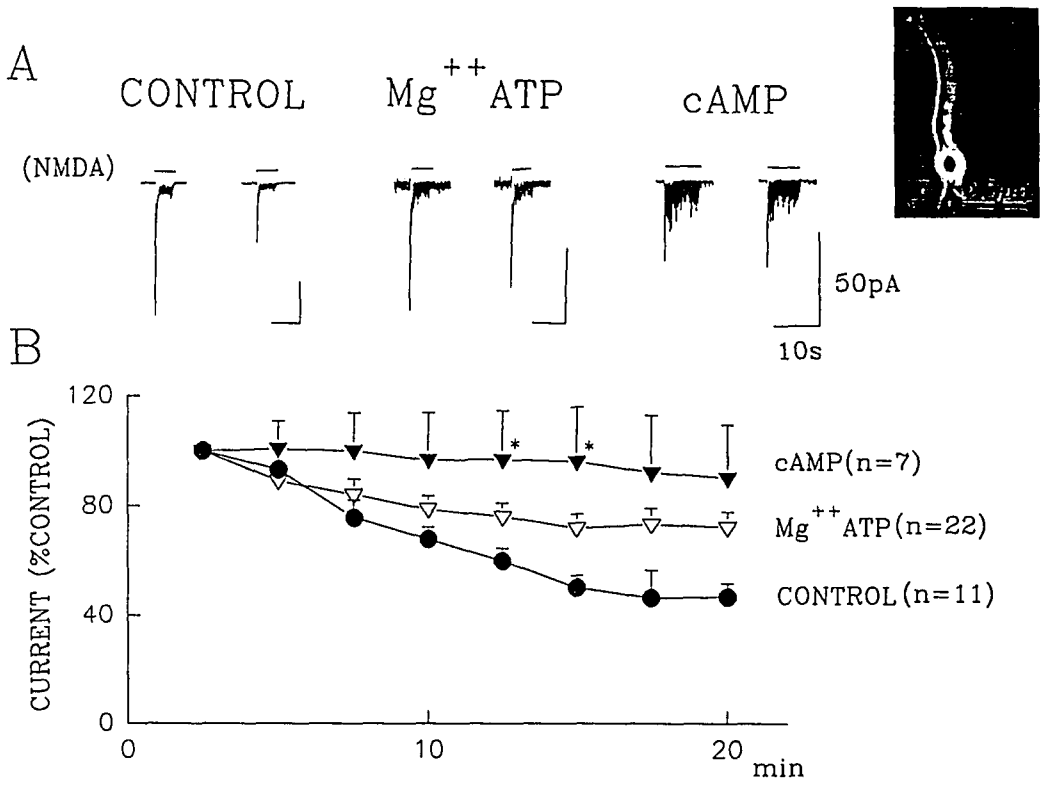
To compare responses between different cells the peak amplitude of EAA-induced inward current at any given time was normalized with respect to the value of first response. Population results are expressed as means ± S.E.M. For statistical analysis we used one way ANOVA and statistical significance between means was determined by Student-Newman-Keuls Test.

RESULTS

In order to examine a possible role of PKA in the regulation of NMDA receptors, we first examined the effects of MgATP and cyclic AMP on the wash-out of NMDA currents. As shown in the previous work (23), and here in Fig. 1 (control curve) the peak amplitude of NMDA currents progressively declined to approximately 50% of their initial amplitude within 15 min of dialysis with an intracellular solution containing CsCl and 1mM EGTA as calcium buffer. The inclusion of 6mM MgATP (Fig. 1A,B) (or in several cells of 3mM MgATP, 0.1mM GTP and 0.1mM leupeptine, a Ca^{2+}-activated neutral protease inhibitor, data not illustrated) in the intracellular medium significantly reduced the time-dependent wash-out of NMDA currents. Moreover, dialysis with a solution containing cyclic AMP (40-80 μM) appears to sustain NMDA currents for 15 min, or more (Fig. 1A,B). These results suggest that phosphorylation, possibly through the cyclic AMP-dependent protein kinase system is required for maintenance of NMDA receptor in the functional state.

In order to determine whether endogenous PKA can modulate NMDA currents, we externally perfused DH neurons with 8-Br cyclic AMP, a membrane permeable analog of cyclic AMP, and also used intracellular perfusion with cyclic AMP. When single DH neurons were exposed to 8-Br cyclic AMP (10-100μM) for 5 min before the testing of NMDA responses, an increase (to 121.8 ± 3.0% of control, n=14, mean ± SEM) in the peak amplitude of the transient component of NMDA-induced current was observed (Fig. 2A,C). On average, the maximum effect occurred 10 min after the

Fig. 1. Stability of peak NMDA currents in acutely isolated spinal dorsal horn neurons in the presence of three different intracellular dialysis solutions. NMDA (100μM, 8s) was applied at regular 2,5 min intervals to neurons held at -60mV (in this and subsequent figures) in a whole-cell voltage-clamp configuration. A; Pairs of typical NMDA-induced current responses obtained at 2.5 min (left trace) and at 20 min (right trace) after the rupture of the patch for three different neurons. The peak amplitude of the NMDA current responses characteristically decreased to 50% of the initial amplitude after the 20 min of dyalisis with CsCl-containing solution (CONTROL). When $Mg^{++}ATP$ (6mM) was added to the control solution, the rundown was significantly reduced ($Mg^{++}ATP$), and almost completely abolished when cAMP (40-80μM) was added to the control solution (cAMP, inset). B; Time course (20 min) of the NMDA-induced current responses for the three different intracellular dialysis solutions. Data are presented as means±SEM. Statistical significance ($P<0.05$) of data is marked by an asterisks. A; 11-day-old rat (left traces), 11-day-old rat (middle traces), 9-day-old rat (right traces, inset). B; 8 to 15-day-old rats.


A
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(NMDA)
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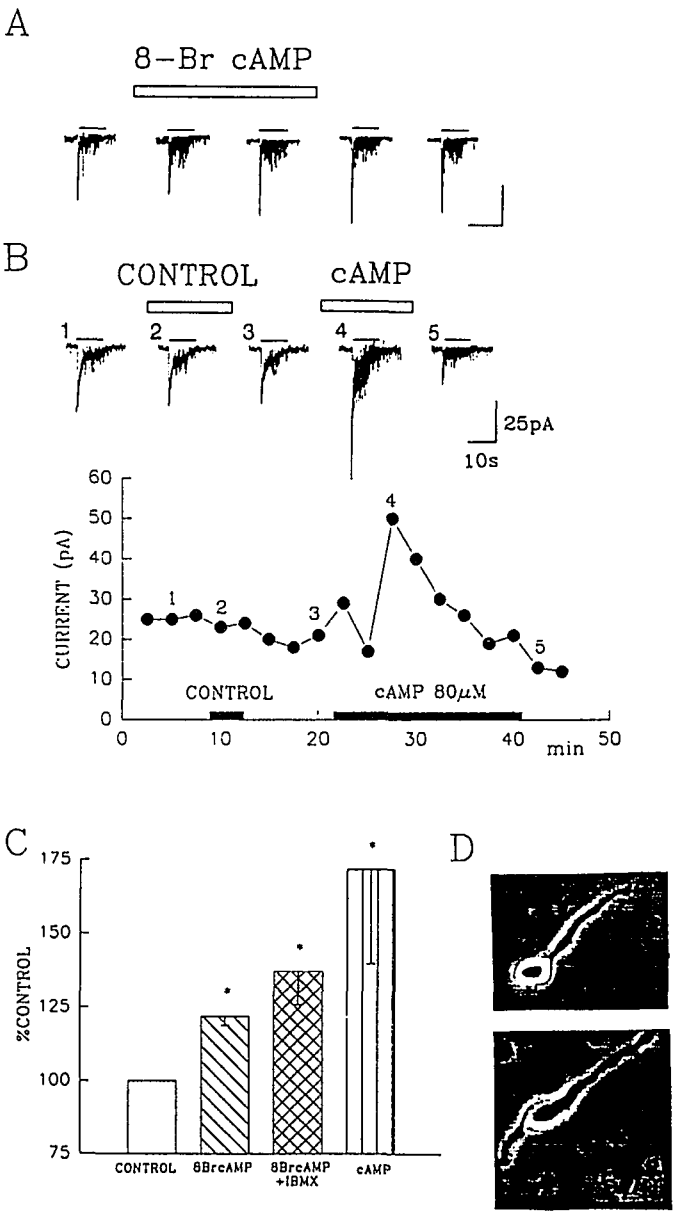
min
cAMP(n=7)
Mg++ATP(n=22)
CONTROL(n=11)

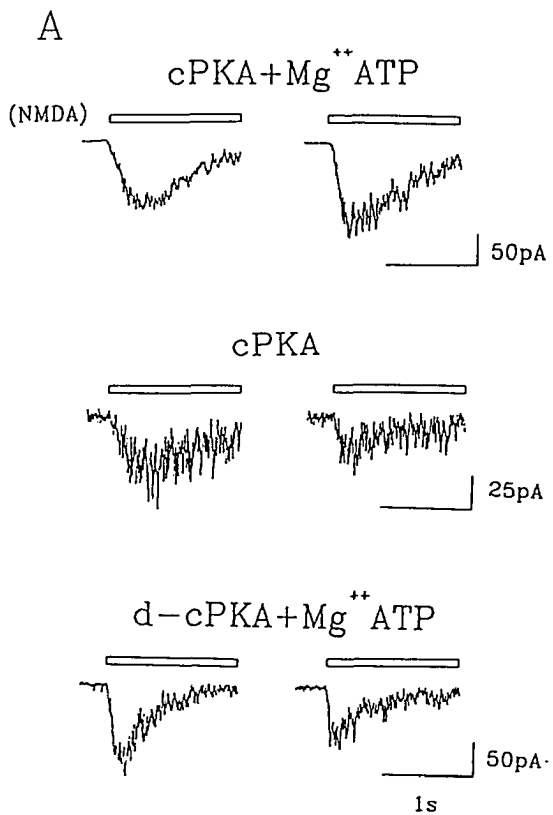
Fig. 2. The effects of 8-Br cAMP and intracellular dialysis with cAMP on NMDA-induced currents recorded from dorsal horn neurons. A; NMDA current responses recorded from a dorsal horn neuron (D; upper inset) before, during and after the pressure aplication of 8-Br cAMP (10μM, 6min). The NMDA response was recorded with a nystatin (550μM/ml) perforated patch technique, and 20 min were allowed, after formation of a seal, for the NMDA responses to stabilize before the start of experiment. B; NMDA currents in a different dorsal horn neuron (D; lower inset) in the presence of regeneration system (Mg^{++}ATP (3mM), GTP (0.3mM) and leupeptin (0.1mM)) in the patch electrode. Time course of the peak NMDA-induced currents before, during and after intracellular perfussion with the control solution or cAMP (80μM)-containing control solution. The individual NMDA responses are illustrated above the plot. C; Summarized results showing the enhancement of NMDA-induced currents produced by 8-Br cAMP (10-100μM), 8-Br cAMP (100μM) + IBMX (0.5mM) or by inracellularly pressure-applied cAMP (80-200μM). A; 12-day-old rat. B; 13-day-old rat. C; 8-15-day-old rats.

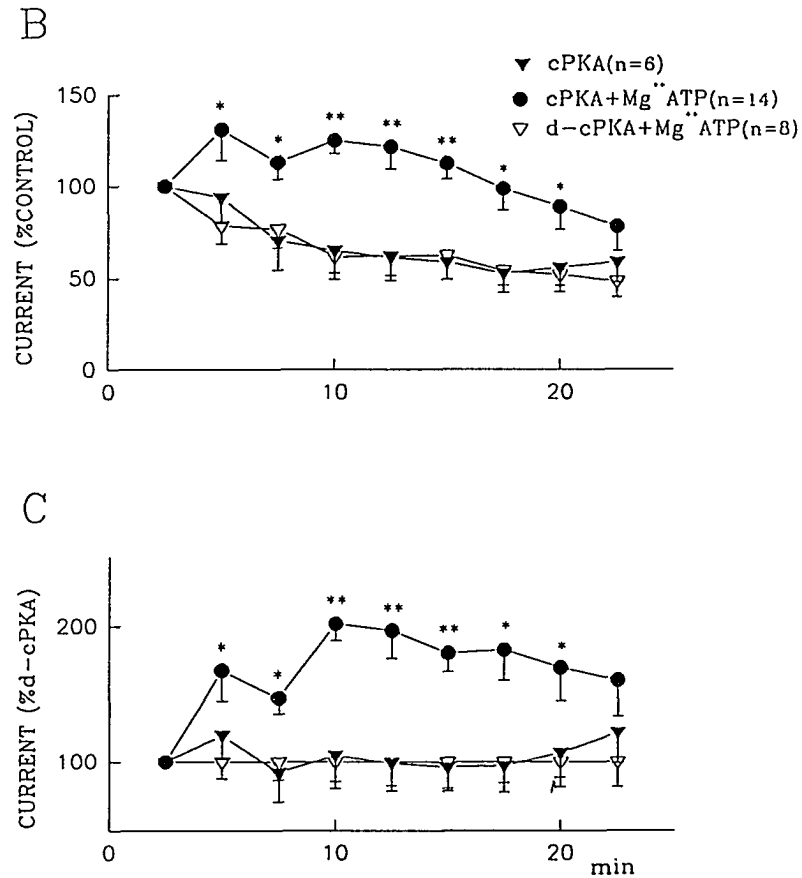

A
8-Br cAMP
B
CONTROL
cAMP
1
2
3
4
5
25pA
10s
CURRENT (pA)
60
50
40
30
20
10
0
CONTROL
cAMP 80μM
0
10
20
30
40
min
50
C
%CONTROL
175
150
125
100
75
CONTROL
8BrcAMP
8BrcAMP +IBMX
cAMP
D

onset of pressure application of 8-Br cyclic AMP. The potentiation was frequently preceded by a depression (by 66.4 ± 5.5%, n=7) of the NMDA response (Fig. 2A). This depressant effect was absent in the presence of 10-100nM added glycine to the recording medium. The potentiating effect of 8-Br cyclic AMP on the NMDA-induced currents was enhanced (to 137.1 ± 11.3%, n=9) when the perfusing medium contained the inhibitor of phosphodiesterase IBMX (0.5 mM; Fig. 2C). Similar potentiation (171.8 ± 41.3%, n=3) was obtained when DH neurons were intracellularly perfused with cyclic AMP (40-200μM) for 6-10 min (Fig. 2B-C). In a cell, illustrated in Fig. 2B, the enhancing effect of cyclic AMP took 2-3 min to appear, 6 min to reach a maximum and declined in 10 min during the perfusion with cyclic AMP. The enhancing effect was absent when the cells (n=5) were exposed only to the superfusate without cyclic AMP (Fig. 2B, control), the finding ruling out the possibility that any dislocation of a cell resulting from intracellular perfusion, may have contributed to the effect.

Since the effect of 8-Br cyclic AMP and cyclic AMP is most likely mediated by activation of protein kinase A we directly tested for this possibility by intracellularly applying catalytic subunit of PKA (cPKA). When internal surface of a cell was exposed to 20μg/ml of cPKA in the presence of 6mM MgATP, the NMDA current increased (n=14) after penetrating the cell reaching a value of 131.3 ± 17.0% of the control (1st NMDA response) after 5 min and then declined slowly to a value of 98.8 ± 11.8% of control after 18 min (Fig. 3A-C). Inclusion of MgATP alone (Fig.1, n=22) or 20μM of heat-denaturated cPKA (d-cPKA) with 6mM MgATP (Fig. 3, n=8) or cPKA without MgATP (Fig. 3, n=6) yielded wash-out statistically not

Fig. 3. The effect of cPKA on NMDA-induced currents. Typical examples of NMDA currents recorded from dorsal horn neurons at -60mV with three different types of internal solutions. A; The left traces correspond to the responses obtained at 2.5 min after, and the right traces 10 min after the rupture of the patch. One of the following substances was added to the standard intracellular dialysis solution: cPKA(20μg/ml)+ Mg^{++}ATP(6mM)(A; upper traces), cPKA(20μg/ml)(A; middle traces) or heat-denaturated cPKA(20μg/ml)+ Mg^{++}ATP(6mM)(A; bottom traces). B; Time course of the mean peak amplitude of NMDA currents for three different intracellular dialysis solutions expressed as percentage of the first(control) response. C; The same data expressed as percentage of d-cPKA response. Statistical difference to d-cPKA: * $P<0.05$, ** $P<0.01$. A; 9-day-old rat (upper trace), 9-day-old rat (middle trace) and 11-day-old rat (bottom trace). B,C; 8 to 15-day-old rats.


A
cPKA+Mg++ATP
(NMDA)
50pA
cPKA
25pA
d−cPKA+Mg++ATP
50pA
1s


B
cPKA(n=6)
cPKA+Mg++ATP(n=14)
d−cPKA+Mg++ATP(n=8)
CURRENT (%CONTROL)
150
100
50
0
0
10
20
C
CURRENT (%d−cPKA)
200
100
0
10
20
min

different from one obtained under control conditions (Fig. 1). The amplitude of the NMDA currents recorded with electrodes containing cPKA + MgATP, and measured 18 min after penetrating the cell, was significantly higher than the currents recorded under control conditions (Fig. 1) or when dPKA + Mg^{2+}ATP or cPKA alone were used (Fig. 3).

DISCUSSION

Our results indicate that NMDA currents of isolated rat spinal DH neurons are modulated by the activity of PKA. Our findings that NMDA-evoked current was potentiated by treatment of these neurons with forskolin (23) or 8-Br cyclic AMP, as well as on the direct perfusion of the cells with cyclic AMP or PKA is consistent with the results obtained in DH neurons using the in vitro spinal slice preparation (1,6), and the observations recently made in Xenopus oocytes injected with rat brain RNA (17). However, our present finding of the enhancement of macroscopic NMDA currents by PKA differs from the results obtained in the hippocampus, where whole-cell and single channel analysis in cultured rat hippocampal neurons revealed no obvious alterations of the NMDA channel properties (7,26). This difference in the results may arise from different experimental protocols used or from differential expression of NMDA receptor subtypes in the two preparations studied. The mechanism underlying the potentiation of NMDA peak current by PKA remains to be determined. Several possibilities could be considered. Thus NMDA receptor-channel complexes might be directly phosphorylated by PKA, or alternatively regulation of the receptor channels could be indirect by means of regulatory proteins associated with channels. The latter possibility appears to be more likely, since the various subunits of the cloned rat NMDA receptor contain consensus phosphorylation sites for Ca^{2+}-calmodulin-dependent protein kinase type II and protein kinase C (12,18), and no consensus sequence for PKA. However, in view of the multiplicity of NMDA receptor subtypes, further study is

needed to determine whether NMDA receptor is directly phosphorylated by known protein kinases. The effect of PKA on NMDA currents of DH neurons could result also from a recruitment of additional receptors (11,15) or by the activation of a different subtype of a receptor.

It is well known that the properties of the nicotinic acetylcholine receptor (nAChR) can be regulated by a variety of pharmacological agents and physiological ligands binding to sites distinct from the neurotransmitter binding sites, referred to as allosteric sites (13). Although the physiological significance of this modulation is not fully elucidated, its potential role in the modulation of synaptic efficacy is suggested. The study of nAChR of ciliary ganglion neurons revealed the presence of a large pool of silent receptors that may be converted into active state through a cyclic-AMP-dependent process (11,15). Alternatively, intracellular nAChRs may become exposed to the surface of the cell in the presence of cyclic AMP (16). Similar to nAchR, NMDA-gated ion channel is also a transmembrane protein carrying multiple binding sites, that may link through the membrane, multiple convergent signals from the outside, or the inside of the membrane, and serve as building components for the "chemical Hebb synapse".

The results obtained in the present work describe the effects of the cyclic AMP second-messenger system on extrajunctional NMDA receptors, rather than those localized in the postsynaptic membrane. To determine if junctional NMDA receptors might also be modulated through the cyclic AMP cascade, it will be necessary in a future work to isolate the NMDA component of the excitatory postsynaptic current and determine whether the

activation of cyclic AMP second messenger system affects the properties (amplitude, decay time) of these currents.

Even though NMDA receptors play a minor role in mediation of the dorsal root-evoked monosynaptic EPSP of DH neurons, NMDA receptors are involved when the pathway was activated at high frequency (6), and are also critically involved in neuronal plasticity such as long-term potentiation (21). Our study suggest that in the rat spinal DH cPKA may be involved in the regulation of the NMDA receptor sensitivity and may contribute to some aspects of postsynaptic plasticity that may play a role in integration of sensory information, including pain. Recent study (25) has suggested that cPKA plays a role as a secondary messenger system in the process of hyperalgesia at the primary afferent synapse.

Acknowledgements

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PAPER III. MODULATION OF AMPA AND NMDA RESPONSES IN RAT SPINAL DORSAL HORN NEURONS BY TRANS-1-AMINOCYCLOPENTANE-1,3-DICARBOXYLIC ACID

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[1]Published as a research paper by R. Cerne and M. Randić. 1992. Neurosci. Lett.

SUMMARY

In freshly isolated spinal dorsal horn (DH) neurons (laminae I-IV) of the young rat, the effects of 25-100μM of (±)-trans-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD), 1S,3R-ACPD and 1R,3S-ACPD, a metabotropic glutamate receptor (mGluR) agonists, on inward currents induced by glutamate (Glu), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate were studied under whole-cell voltage-clamp conditions. When the cells were clamped to -60mV, the racemic mixture and both stereo isomers of trans-ACPD increase the responses elicited by Glu, AMPA, and NMDA, but little those of kainate. In addition, quisqualate (10-50 μM), in the presence of CNQX (5-20 μM) or NBQX (5 μM), potentiated NMDA-induced currents. The enhancing effect lasted 10-75 min, depending upon both dose and length of application. In a smaller proportion of dorsal horn neurons, the enhancing effect was preceded by a transient depression of the responses to Glu, AMPA, and NMDA. 2-Amino-3-phosphono-propionic acid (L-AP_3), a putative antagonist of mGluR exerted little effect on responses to AMPA itself, but reduced or prevented the enhancing effect of 1S,3R-ACPD. It is concluded that activation of a metabotropic glutamate receptor by trans-ACPD, and its two enantiomers, may mediate the enhancement of AMPA and NMDA responses in acutely isolated rat spinal dorsal horn neurons. These results are consistent with the possibility that the activation of metabotropic glutamate receptor may contribute to the regulation of the strength of excitatory amino acid-mediated primary afferent neurotransmission, including nociception.

INTRODUCTION

There are two major classes of receptors for excitatory amino acids (EAA) in the vertebrate CNS that have been termed "ionotropic" and "metabotropic" [19,20]. The ionotropic (AMPA, kainate and NMDA) receptors directly regulate the opening of ion channels to Na^+, K^+ and Ca^{2+}. The metabotropic glutamate receptors (mGluRs) are coupled through a G protein(s) to phosphoinositide-specific phospholipase C and probably present both pre- and postsynaptically [6,17]. The complementary DNA of a mGluR has been recently cloned and characterized [12]. Metabotropic glutamate receptor is activated by the rigid glutamate analog, (±)-trans-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD) [15]; a claimed selective agonist for this receptor being 1S,3R enantiomer of ACPD [9], whereas quisqualate is nonselective but potent agonist at this receptor. There is evidence that activation of the mGluR has a role in the generation of long-term potentiation and long-term depression [2,13]. Although AMPA receptor plays a key role in mediating expression of both forms of plasticity, trans-ACPD has recently been shown to potentiate NMDA but not AMPA responses in hippocampal neurons [1,8]. We now report that (±)-trans-ACPD, and both stereo isomers of trans-ACPD, increase AMPA and NMDA responses in freshly isolated rat spinal dorsal horn (DH) neurons.

METHODS

Single spinal DH neurons in the Rexed's laminae I-IV were isolated acutely from seven to fourteen day-old Sprague-Dawley rats by the method described elsewhere [14]. The whole-cell voltage clamp technique [7] was used to record membrane currents of isolated DH neurons at room temperature (20-23°C). Currents elicited by EAA and holding voltages were monitored continuously with a List L/M-EPC7 patch-clamp amplifier and recorded on a Gould-Brush pen recorder. The solution perfusing the outside of the cell had the following composition (mM): NaCl 150, KCl 5, $CaCl_2$ 0.5 or 2, $MgCl_2$ zero or 1, HEPES 10, D-glucose 10, NaOH to adjust pH to 7.4, bovine serum albumin 0.1 mg/ml, and tetrodotoxin (5×10^{-7}M). Electrodes were filled with one of 2 internal solutions (in mM): K-aspartate 120, KCl 20, NaCl 10, $MgCl_2$ 1, HEPES 10, BAPTA or EGTA 1, NaATP or MgATP 3, GTP 0.1-0.3, leupeptin 0.1 and TRIS base for pH 7.2. In more than half of experiments we replaced K-aspartate (120 mM) and KCl (20 mM) with CsCl (140 mM). EAA-glutamate (GLU), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, N-methyl-D-aspartate (NMDA), 1S,3R-ACPD, 1R,3S-ACPD and a racemic mixture (1S,3R and 1R,3S) of ACPD were dissolved in the HEPES-buffered solution and applied by a pressure system [14]. Only one cell in a dish was subject to one trial with (±)-trans-ACPD, or its enantiomers, the exception being the experiments using 2-amino-3-phosphonopropionate (AP3) where each cell was subjected to two trials with ACPD. EAAs, various forms of trans-ACPD and L-AP_3 were obtained from Cambridge Research Biochemicals and Tocris. To compare responses between different cells the

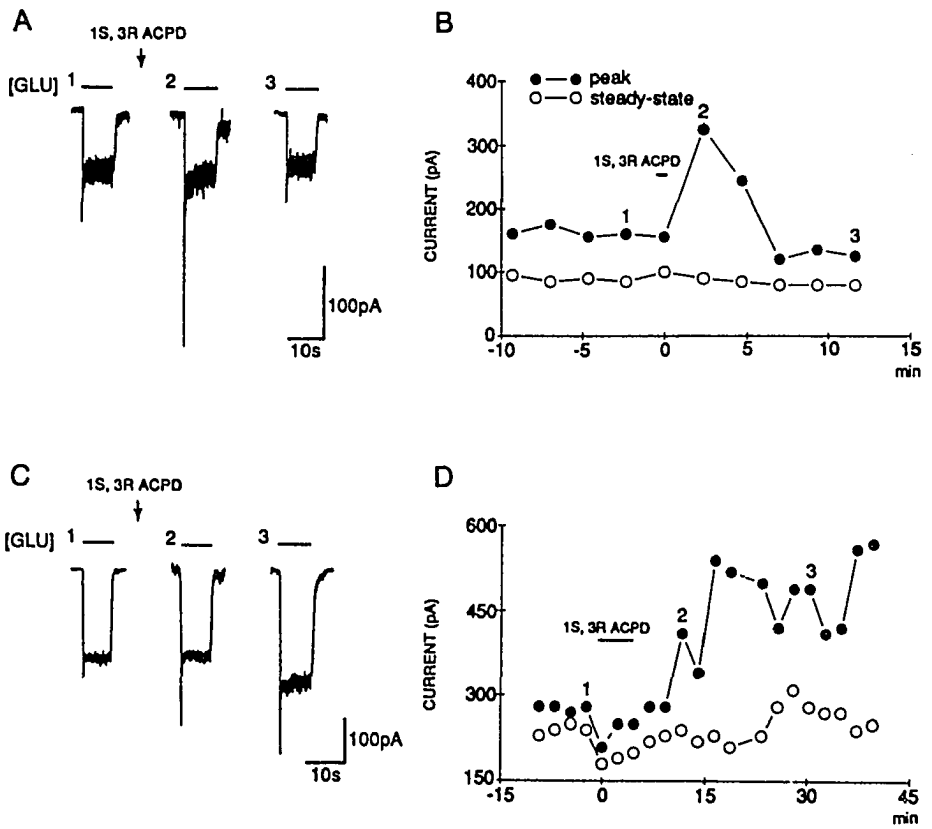
peak and steady-state amplitude of EAA-induced inward current at any given time was normalized with respect to the average values of first three responses recorded prior to trans-ACPD administration. Population results are expressed as means ± S.E.M. For statistical analysis we used one way ANOVA and statistical significance between means was determined by Student-Newman-Keuls test.

RESULTS

To investigate direct postsynaptic interactions between ionotropic and mGluRs we perfused DH neurons with 25-100μM of (±)-trans-ACPD or 1S,3R-ACPD or 1R,3S-ACPD, for either 40-60s or 6 min, and continuously applied EAAs at 2.5 min intervals before, during and after drug addition, for 60 min or more. In most of the experiments 0.5 μM TTX was present in the perfusing solution. When DH neurons were voltage clamped to -60mV, pressure application of GLU (10-30μM), AMPA (10-20μM) or NMDA (30-100μM) for 8-10s induced inward currents that showed fast desensitization (Figs. 1-4) in most of DH neurons [14]. At a holding potential of -60mV, the AMPA responses were relatively constant (Fig. 2A,B) showing an average 10% decrease in the peak amplitude within 60 min of recording. Somewhat greater reduction in the peak amplitude of the NMDA responses occurred during the first 25 min of recording. Thereafter, the NMDA responses stabilized at approximately 75% of the initial amplitude when CsCl-intracellular dialysis solution was used [16].

1S,3R-ACPD (25-100μM) induced a small (range: 1-7 pA) slowly developing and reversible steady inward current that was accompanied by an increase in membrane current noise in 11 of 17 examined cells. As shown in Fig. 1A-B, application of 100μM of 1S,3R-ACPD for 40s caused a reversible increase of the Glu-elicited inward current; the peak amplitude of the fast component increased to 167.3 ± 28.8% of control (n=3). With longer (6 min) pre-treatment of a DH neuron with 1S,3R-ACPD, the enhancing effect lasted > 40 min (Fig. 1D). The enhancing effect was absent when the cell was exposed

Fig. 1. Two different DH neurons were clamped to -60 mV and initial transient and steady-state components of inward current measured at the peak and the end of current excursion generated by 10s applications of 25 μM GLU (A,B) and 30 μM GLU (C,D). Bars denote periods of a rapid application of GLU. A-B: 1S,3R-ACPD, 100 μM, 40s; C-D: 1S,3R-ACPD, 100 μM, 6 min. Time courses of the peak and steady-state GLU current responses recorded before, during and after 1S,3R-ACPD administration are shown in the graphs (B,D). In this, and Figs. 2-4, the intracellular solution contained in mM: 140 CsCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA, pH 7.2. Tetrodotoxin (TTX 0.5μM) was added to the recording solution. The results are expressed as the mean percentage of the three responses evoked by GLU before applying 1S,3R-ACPD. A, B: 12-days-old-rat; C, D: 13 days-old-rat.


A
1S, 3R ACPD
[GLU] 1
2
3
100pA
10s
B
peak
steady-state
1S, 3R ACPD
CURRENT (pA)
400
300
200
100
0
-10
-5
0
5
10
15
min
C
1S, 3R ACPD
[GLU] 1
2
3
100pA
10s
D
1S, 3R ACPD
CURRENT (pA)
600
450
300
150
-15
0
15
30
45
min

only to the superfusate without 1S,3R-ACPD, the finding ruling out the possibility that any dislocation of a cell resulting from flow, may have contributed to the effect. Since Glu activates AMPA, kainate and NMDA receptors in the rat spinal DH neurons [14], we used these selective agonists of Glu receptors in order to determine which receptor subtype 1S,3R-ACPD interacted with.

1S,3R-ACPD (100 μM) caused an increase (40-60s perfusion: to 132.3 ± 10.8%, n=11; 6 min perfusion: to 147.5 ± 11.3%, n=13) in the initial peak amplitude of the transient component of AMPA-induced current in all examined cells (Fig. 2C,F) and also potentiated the steady-state component (40-60s: to 117.3 ± 5.7%; 6 min: to 122.8 ± 5.2%) of the AMPA response (Fig. 2C). The enhancement of AMPA response was in 7 of 27 cells pulsatile in nature. The AMPA-induced currents were increased by 1S,3R-ACPD not only during co-administration but up to 65 min after removal of the drug (Fig. 2D,E) and the effect showed (18 of 24 cells) in part recovery (40s perfusion: to 104.1 ± 2.5%; 6 min: to 117.8 ± 7.6%). However in 10 of 24 cells the potentiation (to 120.8 ± 10.0%) of the AMPA-induced current by 1S,3R-ACPD was preceded by a transient depression (to 86.1 ± 2.7%), as shown in Figs. 2E and 3. The potentiation of the initial transient component (to 125.7 ± 6.6%, n=4) and the steady-state component (to 120.7 ± 6.9%, n=4) of the AMPA-induced current is produced also by 1R,3S-ACPD (50-100μM) enantiomer of trans-ACPD. The AMPA (10^{-5}M) response was initially decreased (to 80.5% control) and later increased (to 126.0 ± 13.1%) during co-administration with (±)-trans-ACPD (10^{-4}M) in 3 cells. Pre-incubation of DH neurons with 2-amino-3-phosponopropionic acid (L-AP_3) (10^{-3} M for 3-9

Fig. 2. A, the traces show inward current responses in a DH neuron evoked by AMPA (10 μM, 10s), recorded at 2.5 min intervals for 100 min, at V_h -60mV. B, shows the time course of the peak and steady state component of AMPA responses; the numbers (1-6) designate the individual responses illustrated in A. C,D, 1S,3R-ACPD (0.1 mM, 6 min), in the came cell potentiated the transient and steady-state component of AMPA (25 μM)-induced current and the effect showed a partial recovery. E, L-AP_3 (10^{-3}M) prevented the enhancing effect of 1S,3R-ACPD. F, summarized results showing the enhancement of AMPA responses (transient component) by 1S,3R-ACPD applied for 40s or 6 min and the antagonism of the effect by L-AP_3. The results are presented as mean ± SEM, statistical significance of data is marked by an asterisk. A-D, 11-day-old-rat; E, 10-day-old-rat; F, 7-16-days-old rats.

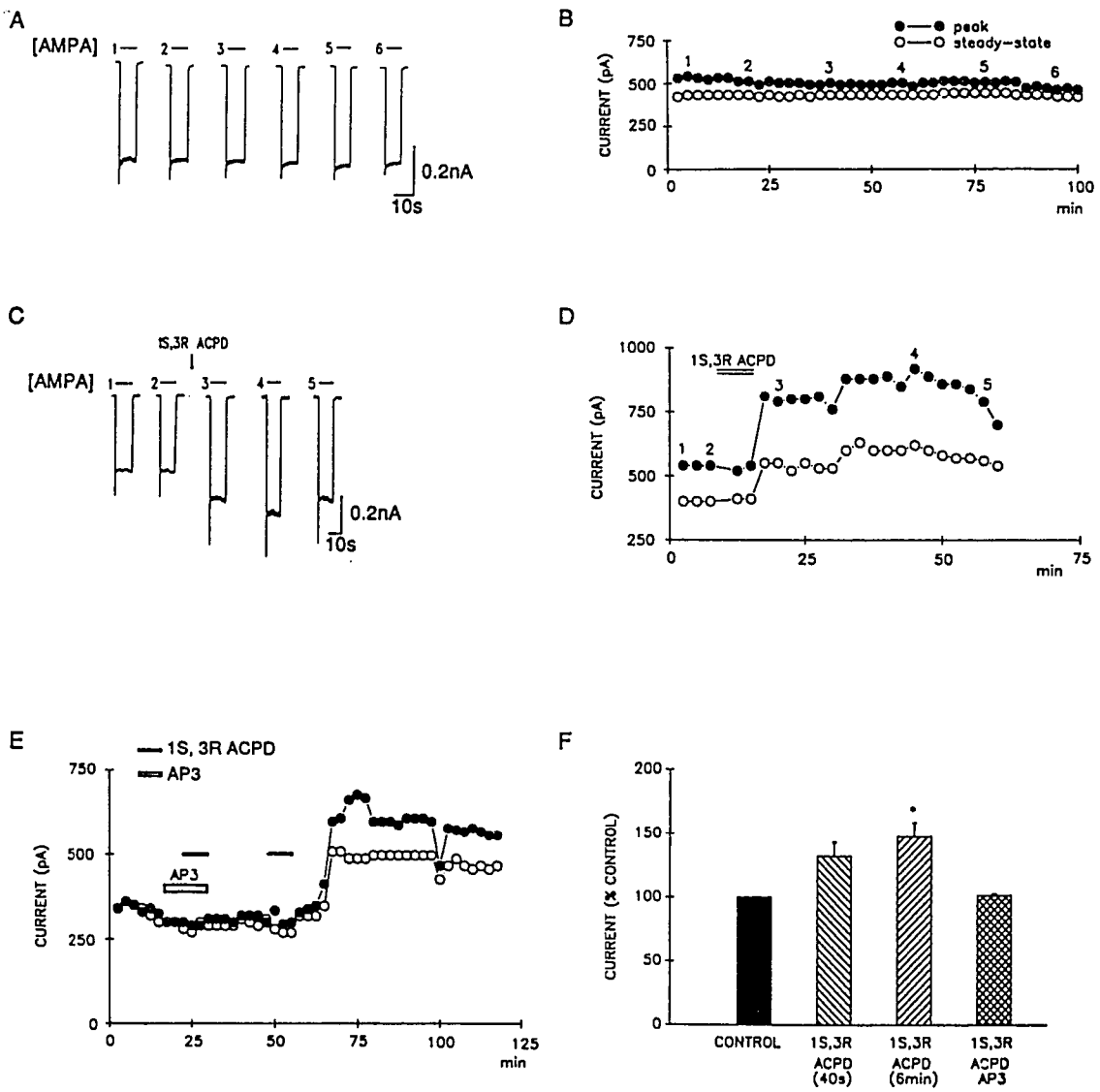
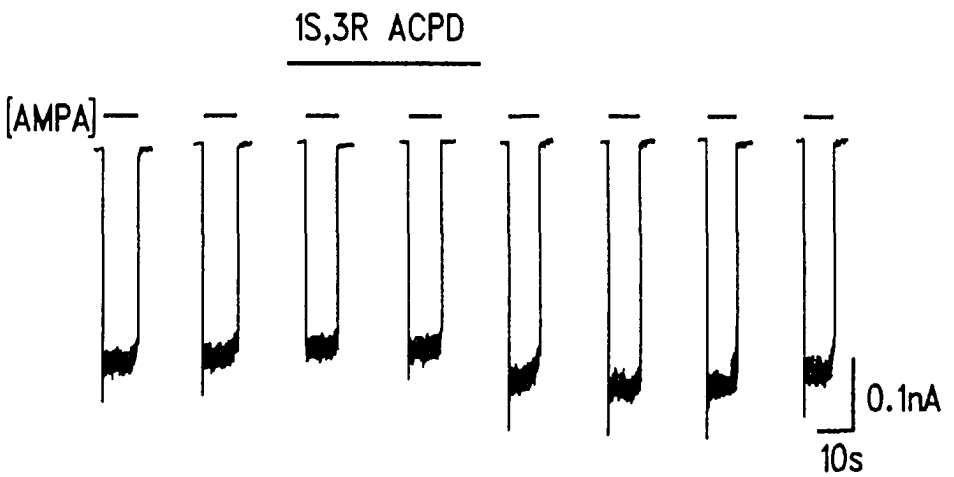

A
[AMPA] 1 2 3 4 5 6
0.2nA
10s
B
peak
steady-state
CURRENT (pA)
750
500
250
0
1 2 3 4 5 6
0 25 50 75 100
min
C
1S,3R ACPD
[AMPA] 1 2 3 4 5
0.2nA
10s
D
1S,3R ACPD
CURRENT (pA)
1000
750
500
250
1 2 3 4 5
0 25 50 75
min
E
1S, 3R ACPD
AP3
CURRENT (pA)
750
500
250
0
AP3
0 25 50 75 100 125
min
F
CURRENT (% CONTROL)
200
150
100
50
0
CONTROL
1S,3R ACPD (40s)
1S,3R ACPD (6min)
1S,3R ACPD AP3

Fig. 3. 1S,3R-ACPD (100 μM, 6 min) initially abolished the transient component of the AMPA (20 μM, 10s)-induced current. The depressant effect was present during the co-administration of the drugs and was followed by a small potentiation of AMPA responses.

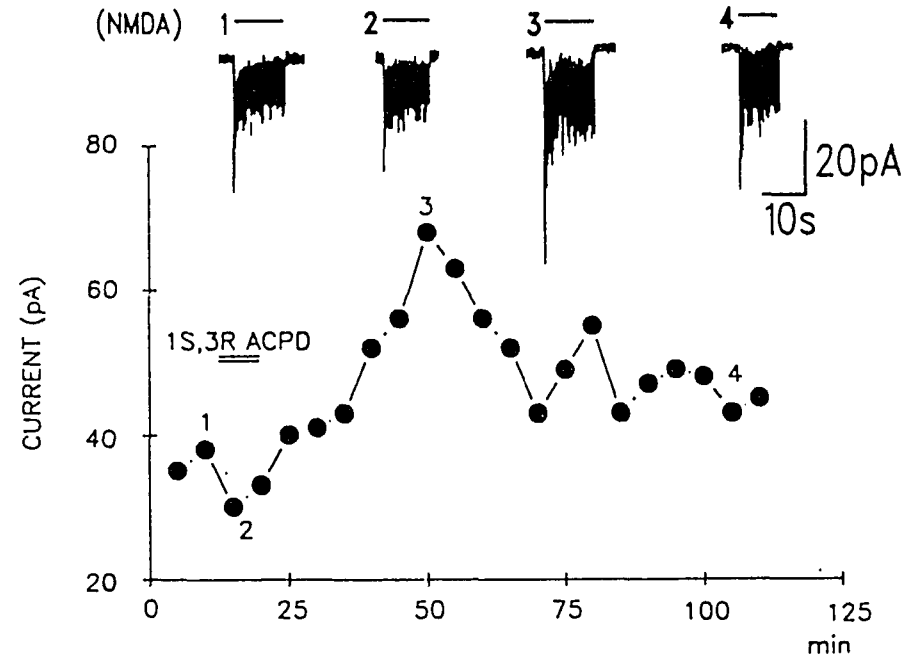

1S,3R ACPD
[AMPA]
0.1nA
10s

min), a putative antagonist of mGluR, exerted little effect on responses to AMPA itself, but prevented (n=4) the enhancing effect of 1S,3R-ACPD (Fig. 2E). Kainate (10-25μM) current is little (decrease to 90%, n=3; increase to 120%, n=1) affected by 1S,3R-ACPD (100μM for 6 min) in 5 cells.

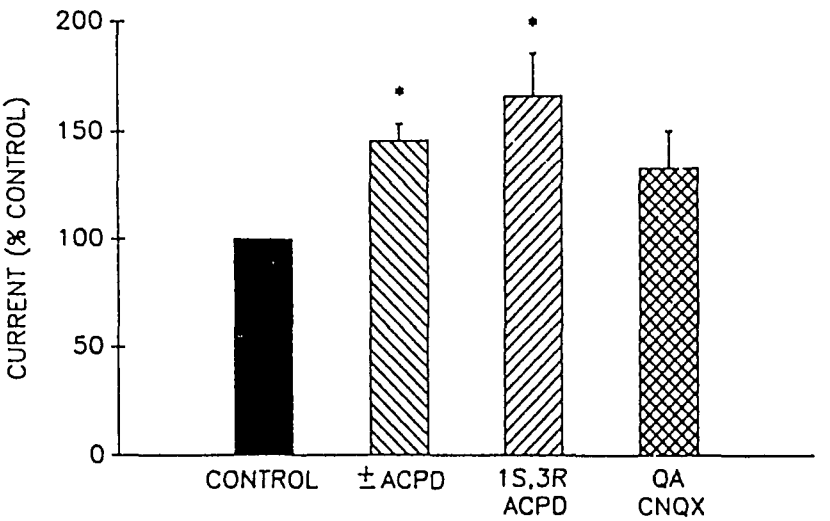
Next, we have examined the effects of a racemic form of trans-ACPD and 1S,3R-ACPD on the NMDA-induced current responses of DH neurons. When the microelectrode solution contained high K^+ with 1mM-EGTA or BAPTA (no added Ca^{2+}), and Mg^{2+} was omitted from a nominally glycine-free perfusing solution containing 0.5μM TTX, pressure application of (±)-trans-ACPD (10-100μM for 2-4 min) enhanced (Fig. 4B) the peak amplitude of the initial transient component of the NMDA-induced current (to 146.1 ± 7.8%) in 11 of 12 tested cells. The enhancing effect of (±)-trans-ACPD was also present under condition of superfusion with glycine (50 nM)-enriched solution. The maximum increase was generally observed about 10 min following the onset of (±)-trans-ACPD application. In 4 of the cells (±)-trans-ACPD produced two distinct effects on the transient component of NMDA-induced current consisting of an initial depression (to 76.5 ± 8.3% of control) followed by a potentiation (to 124.3 ± 4.5%). The dual effect (decrease: to 70.0 ± 5.8; increase: to 166.3 ± 19.5%) was also observed in each of the 4 cells exposed to 1S,3R-ACPD (100 μM for 6 min) and when K-aspartate and KCl in the microelectrode solution were replaced by CsCl (Fig. 4A). The maximum potentiation was observed within 25-30 min following the onset of 1S,3R-ACPD application. Similar as for the AMPA responses, the NMDA-induced currents were modified by trans-ACPD and 1S,3R-ACPD not only during co-administration but up to 75 min after removal of the drugs.A specific

Fig. 4. A, The graph shows the time course of the peak transient component of NMDA (100 μM, 10s) current responses recorded at 2.5 min intervals before, during and after 1S,3R-ACPD (100 μM, 6 min) application. Individual responses are illustrated above the graph. B, summarized results showing the enhancement of the transient component of NMDA-induced current produced by (±)-trans-ACPD, 1S,3R-ACPD and QA co-administered with CNQX. A, 10-day-old-rat; B, 7-15-day-old-rats.

A


(NMDA)
1
2
3
4
20pA
10s
80
60
40
20
CURRENT (pA)
1S,3R ACPD
1
2
3
4
0
25
50
75
100
125
min

B


200
150
100
50
0
CURRENT (% CONTROL)
*
*
CONTROL
±ACPD
1S,3R ACPD
QA CNQX

activation of mGluR can be obtained using quisqualate (QA) in the presence of CNQX or NBXQ, the ionotropic receptors antagonists. As shown in Fig. 4B, QA (10-50 μM for 1 min) in the presence of CNQX (5-20 μM) or NBQX (5 μM) caused a reversible increase (to 130.3 $\pm$ 17.3%) in the peak NMDA response in all cells examined (n=7).

DISCUSSION

In the present investigation we have demonstrated that (±)-trans-ACPD and its enantiomers (1S,3R-ACPD and 1R,3S-ACPD) enhance both the AMPA- and NMDA-induced responses of a subpopulation of freshly isolated spinal DH neurons from young rats. Our finding that (±)-trans-ACPD and 1S,3R-ACPD generate a slow inward current and increase the NMDA responses in DH neurons is in agreement with recent reports [1,8] which showed that 1S,3R-ACPD enhances responses of CA1 pyramidal neurons to NMDA in the hippocampal slice preparation. However, our observation that 1S,3R-ACPD also enhances the AMPA responses of isolated DH neurons contrasts with the results of the previous studies [1,8] which demonstrated that the drug selectively potentiates responses to NMDA, but not AMPA. Although reasons for this difference in the results are presently unknown, they could be due to differences in the cell type and age of the animals used.

In addition, the present findings show that 1S,3R-ACPD produces dual effects, an initial brief depression followed by a prolonged potentiation of the AMPA-and NMDA-induced current responses of a proportion of DH neurons. The ACPD-induced depression of the inward currents could occur as a consequence of antagonism of AMPA or NMDA receptors, activation of a postsynaptic receptor which modifies membrane resistance, or activation of a presynaptic receptor if the cells are incompletely isolated from presynaptic elements. Given the pharmacological profile of 1S,3R-ACPD, antagonism of postsynaptic EAA receptors is unlikely [15,17]. Since the activation of the metabotropic receptor is capable of reducing K^+ currents

[5], the resulting increase in neuronal resistance may contribute to the ACPD-induced depressions of the EAA responses.

The exact molecular mechanism(s) underlying the modulation of AMPA- and NMDA-receptors-activated conductances by (±)-trans-ACPD and 1S,3R-ACPD have yet to be elucidated. Although 1S,3R-ACPD has been proposed as a selective agonist for mGluR, this is still a question of debate. However, our findings that QA in the presence of CNQX or NBQX also enhances NMDA responses of DH neurons and that L-AP_3 prevented the enhancing effect of 1S,3R-ACPD on AMPA responses support this possibility. The major effects associated with mGluR(s) activation has been stimulation of IP_3 synthesis [17] and mobilization of Ca^{2+} from intracellular stores in neurons [6,9,17]. Changes in $[Ca^{2+}]_i$ can lead to activation of Ca^{2+}-dependent protein kinases or phosphatases resulting in changes in the concentrations of several second messengers and protein phosphorylation. Electrophysiological studies have demonstrated that metabotropic receptor may play an important role in regulation of neuronal excitability and synaptic transmission in the brain. Generation of a slow depolarization [5,18] or an inward current [5,8], activation of a hyperpolarizing response [8], depression of excitatory [3,11] and inhibitory postsynaptic currents have been reported. In addition, trans-ACPD enhances tetanus-induced short- and long-term potentiation (LTP) in the hippocampus [2,13]. Although a great deal is known about LTP in hippocampus the existence of a similar synaptic plasticity at primary afferent synapses in the rat superficial spinal DH has been only recently demonstrated [10]. This effect in the spinal DH may be at least in part due to ACPD potentiating

the responses of DH neurons to AMPA and NMDA. However, until specific antagonists for ACPD receptors are developed, we can only speculate about the possible roles of ACPD receptors in synaptic transmission and plasticity in the rat spinal DH.

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GENERAL DISCUSSION

The major points pertaining to the results presented in this thesis have already been discussed in the "Results". This chapter will outline the major conclusions derived from the presented data and offer some speculative ideas related to the mechanisms underlying our experimental findings.

Several lines of evidence suggest the involvement of second messenger systems in the modulation of excitatatory amino acid mediated synaptic transmission at the level of spinal dorsal horn:

- Spinal dorsal horn is the area where primary afferent fibers terminate and make first synapse with dendrites of dorsal horn neurons. For this reason it is regarded as an important site of initial processing and integration of sensory information derived from skin, viscera and muscles.
- Glutamic acid is the major candidate for the neurotransmitter mediating fast excitatory synaptic transmission of primary sensory neurons including C-nociceptive fibers (Mayer and Westbrook, 1987, Gerber and Randic, 1989a,b, Kangrga and Randic, 1990, 1991, Gerber et al., 1991)
- Primary afferent fibers, descending fibers and interneurons in the dorsal horn contain several neuropeptides (Hökfelt et al., 1975, Kanazava et al., 1984, Urban and Randic, 1984, Jessel and Dodd, 1989, Seybold et al., 1989) that can be released upon stimulation and act on receptors coupled to second messenger systems (Schwartz and Kandel, 1991). In addition to neuropeptides at least two subtypes of glutamate receptor, NMDA (Cvetkovitch et al., 1991) and metabotropic (Sladecek et al., 1985,

Sugiyama et al., 1987, Tanabe et al., 1992, Schoepp and Conn, 1993), are capable of activation of second messenger system.

- Second messenger systems are localized in spinal dorsal horn neurons Worley et al., 1986, Mochley-Rosen et al., 1987, Saito et al., 1988, Mori et al., 1990).
- The neuroplasticity phenomena such as LTP and LTD that involve activation of second messenger systems and were previously described in the higher portions of the central nervous system exist also at the level of the spinal dorsal horn (Randic et al., 1993).
- It is likely that the modulation of excitatory amino acid-mediated neurotransmission by second messengers underlies the modulation of synaptic transmission in the spinal dorsal horn and contributes to phenomena such as primary and secondary hyperalgesia (Hardy et al., 1952, Campbell et al., 1989).

This research has examined two of the possible modulatory systems that may control synaptic efficacy in the spinal dorsal horn, the adenylate cyclase-cyclic AMP-dependent second messenger system and the second messenger-coupled metabotropic glutamate receptor; that will be discussed separately.

The activation of the cyclic AMP-dependent second messenger system enhances synaptic and EAA-induced responses of rat spinal dorsal horn neurons

When dorsal root electrical stimulation was used to elicit excitatory postsynaptic potentials from the substantia gelatinosa neurons, the

activation of the cyclic AMP-dependent second messenger system induced a potentiation of presumably monosynaptic EPSPs. This potentiation was often associated with a small depolarization of resting membrane potential and increase in the frequency of spontaneous postsynaptic potentials. The latter data were consistent with the results obtained in the hippocampal CA1 pyramidal neurons (Chavez-Noriega and Stevens, 1992) and locus coeruleus neurons (Wang and Aghajanian, 1990) and differ from some of the previous studies showing that the activators of cyclic AMP-dependent second messenger system caused inhibition of spontaneous activity and hyperpolarization of CA1 cell membrane potential (Segal, 1981).

In principle, the mechanism of the observed enhancement of the fast excitatory synaptic transmission by the cyclic AMP-dependent second messenger system can be: 1) presynaptic, i.e. the consequence of an increased release of neurotransmitter(s) from presynaptic terminals; 2) postsynaptic, resulting from the enhanced responsiveness of neurotransmitter receptors to released neurotransmitters or 3) it can be due to increased interneuronal activity. Our finding that 8-Br cyclic AMP increases the frequency of spontaneous EPSPs is consistent with the possibility that cyclic AMP enhances presynaptic release of neurotransmitters. This result confirms the results obtained in the previous studies demonstrating that cyclic AMP induces enhancement of presynaptic release of neurotransmitters from DRG terminals (Crain et al., 1986, Shen and Crain, 1989). Similar enhancement of neurotransmitter release by cyclic AMP has been also reported in hippocampal and *Aplysia* neurons and seem to represent a general mechanism of regulation of synaptic

efficacy by cyclic AMP-dependent second messenger systems (Castellucci et al., 1980, 1982, Greengard et al., 1991, Chavez-Noriega and Stevens, 1992). In addition to the effect at the presynaptic site, the cyclic AMP-induced enhancement of fast EPSPs in the spinal dorsal horn may be a consequence of the increased sensitivity of postsynaptic glutamate receptors. When the dorsal horn neurons were pharmacologically isolated from presynaptic input by TTX, the activators of cyclic AMP-dependent second messenger system enhanced the depolarizing responses to bath-applied NMDA, AMPA, KA and QA in more than half of the examined neurons. However, use of intracellular voltage recordings from a relatively intact slice preparation, and the fact that the membrane potential is the final common output of a number of presynaptic and postsynaptic processes, makes it difficult task to assign conclusively a locus or mechanism to the effects produced by bath application of cyclic AMP analogues. To avoid these difficulties, we utilized in the subsequent studies the freshly isolated dorsal horn neurons from young rats and the whole-cell voltage-clamp technique. The fact that under these conditions the activation of the cyclic AMP-dependent second messenger system by 8-Br cyclic AMP enhanced AMPA- and NMDA-induced current responses of DH neurons, confirmed the direct postsynaptic action of the cyclic AMP-dependent second messenger system. In addition, the effect on NMDA-induced currents was reproduced by the intracellular application of cyclic AMP and catalytic subunit of protein kinase A. The study suggested direct phosphorylation of NMDA receptor channel proteins by PKA as a possible mechanism. As an alternative possibility, we can not rule out phosphorylation of regulatory proteins associated with the NMDA receptor-

ion channels.

The results obtained in white perch retinal horizontal cells, mammalian hippocampal neurons, and GluR6 glutamate receptor transiently expressed in mammalian cells confirmed our observation that non-NMDA glutamate receptors can be regulated by cyclic AMP-dependent protein phosphorylation (Liman et al., 1989, Greengard et al., 1991, Wang et al., 1991, Chavez-Noriega and Stevens, 1992, Raymond et al., 1993). It is likely that non-NMDA receptor subtype is directly phosphorylated, since at least one (GluR6) of newly cloned non-NMDA glutamate receptor subunits (Gasic and Hollmann, 1992) contains a major consensus phosphorylation site for phosphorylation by PKA. This possibility was confirmed in recent studies on GluR6 expressed in mammalian cells (Raymond et al., 1993, Wang et al., 1993). The studies demonstrated that a site-specific mutation of a single amino acid (ser 684) in the consensus phosphorylation site of GluR6 reduces or abolishes the enhancing effect of cPKA on glutamate-induced currents. In addition to currents mediated by GluR6, protein kinase A enhances kainate currents of glutamate receptor subunits lacking consensus phosphorylation sequence (Keller et al., 1992). This suggests that protein kinase A can act at additional, low affinity phosphorylation sites (Edelman et al., 1987, Kennelly and Krebs, 1991). Since the affinity of a protein phosphorylation site depends not only on the primary sequence, but also on higher order structures, supplemental experiments, such as site-directed mutagenesis, would be required to evaluate this possibility. Additional studies would be also required in order to establish the mechanism underlying the potentiation of non-NMDA responses by PKA. The studies on GluR6 expressed

in mammalian cells revealed no alteration of properties of glutamate currents, including reversal potential, dose-response relationship, I-V relationship, rise time and desensitization kinetics (Raymond et al., 1993, Wang et al., 1993). The single-channel analysis of non-NMDA currents in hippocampal neurons, however, revealed the PKA-mediated increase in the opening frequency and the mean open time of the non-NMDA channels (Greengard et al., 1991).

In contrast to AMPA, possible modulation of NMDA receptor-channels by PKA remains controversial and less understood (Chen and Huang, 1991, Greengard et al., 1991, Randic et al., 1991, Wang et al., 1991, Cerne et al., 1992, McVaugh and Waxham, 1992). Although the modulation of the NMDA responses of rat DH neurons and oocytes injected with rat brain messenger RNA by protein kinase A (Cerne et al., 1992, McVaugh et al., 1992) has been demonstrated, whole-cell (Wang et al., 1991) and single-channel analysis (Greengard et al., 1991) revealed no obvious alterations of the NMDA channel properties in cultured hippocampal neurons. The difference in obtained results is not understood and it can result from different expression of NMDA subunits in the experimental models used. Contrary to the potentiation of the AMPA-induced currents by PKA, the direct phosphorylation of NMDA receptor by PKA is less likely, since the various subunits of the cloned NMDA receptor contain phosphorylation sites for Ca^{++}-calmodulin-dependent protein kinase type II and protein kinase C (Morioshi et al., 1991, Kutsuwada et al., 1992) and no consensus sequence for PKA. However, in view of the multiplicity of NMDA receptor subtypes, and possibility of phosphorylation at low affinity phosphorylation sites,

further study is needed to determine whether NMDA receptor is directly phosphorylated by known protein kinases. The effect of PKA on NMDA-induced current of DH neurons could result also from phosphorylation of regulatory proteins associated with the channels or recruitment of additional receptors (Margiotta et al., 1987, Knox et al., 1992). It is well known that the properties of the nicotinic acetylcholine receptor (nAChR) can be regulated by a variety of pharmacological agents, and that physiological ligands can bind to sites distinct from the neurotransmitter binding sites, referred to as allosteric sites (Lena and Changeux, 1993). Although the physiological significance of this modulation is not fully elucidated, its potential role in the modulation of synaptic efficacy is suggested. The study of nAChR of ciliary ganglion neurons revealed the presence of a large pool of silent receptors that may be converted into active state through a cyclic-AMP-dependent process (Margiotta et al., 1987, Knox et al., 1992). Alternatively, intracellular nAChRs may become exposed to the surface of the cell in the presence of cyclic AMP (Margiotta et al., 1989). Similar to nAchR, NMDA-gated ion channel is also a transmembrane protein carrying multiple binding sites, that may link through the membrane multiple convergent signals from the outside, or the inside of the membrane, and serve as building components for the "chemical Hebb synapse".

As we evaluate our results and relate the changes in EAA responses to changes in responses to afferent stimulation we should also keep in mind that pressure application of the glutamate receptor agonists, besides activating junctional glutamate receptors, also activate extrajunctional receptors. The latter receptors may be regulated differently (Bekkers and

Stevens, 1989). Whereas activation of PKA may indeed enhance sensitivity of the postsynaptic membrane of DH neurons to exogenous applied glutamate receptor agonists, different mechanisms may account for the increased amplitude of the monosynaptic excitatory postsynaptic potentials. However, the study of synaptic events in hippocampus (Greengard et al., 1991) indicated that forskolin acting through PKA, increases the amplitude and decay time of spontaneous excitatory postsynaptic currents, suggesting the modulation of junctional glutamate receptors.

Our results suggest that in the rat spinal dorsal horn the activation of the cyclic AMP-dependent second messenger system may be involved in the regulation of the sensitivity of postsynaptic excitatory amino acid receptors and primary afferent neurotransmission. It can play an important role in the neuroplasticity phenomena such as long-term potentiation (Cerne et al., 1992, Randic et al., 1993) that was recently described in the spinal dorsal horn and can underlie prolonged changes in computational characteristics of primary afferent synapse. Recent results have suggested that the cyclic AMP-dependent second messenger system plays a role in the decrease of nociceptive threshold (or hyperalgesia) produced by the agents acting on primary afferent terminals (Taiwo and Levin, 1991, Taiwo et al., 1989, 1992).

Modulation of AMPA and NMDA responses in rat spinal dorsal horn neurons by trans-1-aminocyclopentane-1,3-dicarboxylic acid

In freshly isolated spinal dorsal horn neurons (laminae I-IV) of the young rat, studied under whole-cell voltage-clamp conditions, the activation of metabotropic glutamate receptor with trans-ACPD [(±)-trans-1-aminocyclopentane-1,3-dicarboxylic acid] and its enantiomeres 1S,3R-ACPD and 1R,3S-ACPD generated a slow inward current and enhanced inward currents induced by glutamate, AMPA and NMDA.

Our finding that activation of metabotropic glutamate receptor generates slow inward current in dissociated dorsal horn neurons is in agreement with previous studies (Stratton et al., 1989, Charpak et al., 1990, Charpak and Gheweiler, 1991, Zheng and Gallagher, 1991, 1992). The inward current might be due to a metabotropic glutamate receptor induced reduction of voltage-dependent and Ca^{++}-dependent K^{+} conductances (Stratton et al., 1989, Charpak et al., 1990, McCormick and von Krosig, 1992) or to an increase in Ca^{++} conductances (Lester and Jahr, 1990). The exact mechanism of the inward current in our preparation, however, remains to be elucidated.

The enhancing effect of activation of metabotropic glutamate receptor on NMDA-induced current responses, observed in our preparation, is in agreement with recent observations in hippocampal CA1 neurons, spinal dorsal horn neurons and oocytes injected with rat brain RNA (Aniksztejn et al., 1991, Harvey et al., 1991, Bleakman et al. 1992, Cerne and Randic, 1992). However, our observation that 1S,3R-ACPD enhances the AMPA responses of isolated DH neurons contrasts the results of previous studies in

hippocampal CA1 neurons, and oocytes injected with rat brain RNA which demonstrated no significant modulation of AMPA responses by the activation of the metabotropic glutamate receptor (Aniksztejn et al., 1991, Harvey et al., 1991). Our results also differ from results obtained in cerebellar Purkinje cells, where activation of mGluR produces depression of AMPA-induced responses (Linden et al., 1991). Although reasons for this difference in the results are presently unknown, they could be due to differences in the AMPA receptor subtypes expressed in different preparations used, since studies utilizing the acutely dissociated rat dorsal horn neurons and nucleus tractus solitarius neurons confirmed our observation that activation of mGluR enhances AMPA-induced conductances (Bleakman et al., 1992, Glaum and Miller, 1993). In addition, a recent study in the hippocampal CA3 area suggested that the activation of metabotropic receptor in the presence of NMDA inhibitor APV, and in the absence of electrical stimulation, can induce long term potentiation of AMPA mediated component of EPSP (Bartolotto and Collingridge, 1993).

The exact molecular mechanism underlying the modulation of AMPA and NMDA receptor-activated conductances by activation of metabotropic glutamate receptor have yet to be elucidated. Metabotropic glutamate receptor can act through activation of phospholipase C, resulting in production of inositol 1,4,5-triphosphate (IP_3) that releases Ca^{++} from intracellular stores, and diacylglycerol that activates protein kinase C (Sladeczek et al., 1985, Sugiyama et al., 1987). A study in the rat spinal dorsal horn preparation showed that activation of protein kinase C by phorbol esters enhances fast excitatory synaptic transmission and NMDA, QA and AMPA-induced responses of

dorsal horn neurons (Gerber et al., 1989). The enhancement of NMDA conductances by protein kinase C was later confirmed in several other studies (Kelso et al., 1992, Chen and Huang, 1990) and has been suggested as a possible pathway of the mGluR-mediated enhancement of NMDA responses (Kelso et al., 1992, Bortolotto and Collingridge, 1993). In addition recent study demonstrated that the catalytic subunit of protein kinase C enhances AMPA-induced currents and can therefore possibly mediate also the potentiating effect of metabotropic receptor activation on AMPA responses of DH neurons (Cerne and Randic, 1993). Several of the newly cloned AMPA and NMDA receptor subtypes contain consensus sequences for PKC substrates (Gasic and Hollmann, 1992), suggesting direct phosphorylation as a possible mechanism. However, PKC could phosphorylate another protein that directly or indirectly increases ion permeability of AMPA and NMDA receptors-channels. Alternatively, the increase in NMDA- and AMPA-induced currents can be a consequence of the mobilization of Ca^{++} from intracellular stores in neurons (Furuya et al., 1989, Irving et al., 1990, Scheopp et al., 1990). Changes in intracellular Ca^{++} can lead to activation of Ca^{++}-dependent protein kinases or phosphatases resulting in changes in several different second messengers and protein phosphorylation (Xia et al., 1991, Dermot et al., 1993). One of the Ca^{++}-dependent protein kinases, Ca^{++} Calmodulin-dependent protein kinase type II have been recently shown to enhance AMPA induced currents (McGlade-McCulloh et al., 1993). In addition metabotropic glutamate receptor can act also through the activation of cyclic AMP-dependent second messenger system (Tanabe et al., 1992). Cyclic AMP-dependent second messenger system activates protein kinase A, that can

in turn phosphorylate receptors or receptor associated proteins. Both AMPA and NMDA receptors are modulated by protein kinase A (Greengard et al., 1991, Wang et al., 1991, Cerne et al., 1993).

Through induction of prolonged changes in membrane potential and AMPA and NMDA conductances, metabotropic glutamate receptor can induce prolonged changes in synaptic function. Even though there is no evidence demonstrating direct involvement of metabotropic receptor in the generation of LTP in the spinal dorsal horn the studies of a similar phenomenon in the hippocampus demonstrated that the activation of metabotropic receptor enhances tetanus-induced short- and long-term potentiation and can also induce long-term potentiation in the absence of tetanic stimulation (Bortolotto and Collingridge, 1993, Bashir et al., 1993). Any long term change in the computational characteristic of primary afferent synapse may be of relevance for modulation of sensory information processing in the DH.

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