ACTIVITY-DEPENDENT REGULATION OF ∝-AMINO-3-HYDROXY-5-METHYL-
4-ISOXAZOLE PROPIONIC ACID (AMPA) RECEPTORS IN RAT NEOCORTEX

by

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ABSTRACT

The study of α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) receptors is of great interest given that these receptors mediate most fast excitatory synaptic neurotransmission in brain and are involved in neuroplastic phenomena such as long-term potentiation and long-term depression. Understanding the molecular mechanisms involved in the regulation of AMPA receptors may therefore provide insight into many aspects of neuronal function, including normal synaptic transmission and synaptic neuroplasticity. To assess activity-dependent regulation of cortical AMPA receptors, radioligand binding methods employing the competitive AMPA receptor antagonist [^{3}H]-6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were used to study the effects of various regulatory stimuli on the agonist binding site of AMPA receptors in rat cortical slices.

AMPA receptor regulation was studied in response to a variety of stimuli, including agonist (AMPA), pharmacological depolarization (veratridine + glutamate), as well as the phosphorylating enzymes calcium (Ca^{2+}) /calmodulin-dependent kinase II (CaMKII) and protein kinase A (PKA). Treatment with AMPA or veratridine led to approximately 20% decreases in [^{3}H]-CNQX binding. Similar decreases in [^{3}H]-CNQX binding were seen following treatment with CaMKII (~35%) and PKA (~30%).

The effects of AMPA and veratridine could be blocked by inhibitors of CaMKII and PKA, suggesting that phosphorylation reactions are involved in AMPA receptor regulation by AMPA and veratridine. Moreover, loperamide, a non-specific inhibitor of voltage-gated Ca^{2+} channels was able to inhibit the AMPA-

and veratridine-induced regulation of AMPA receptors. These results suggested that AMPA and veratridine may result in the activation of voltage-gated Ca^{2+} channels, in turn leading to changes in $[^3H]$-CNQX binding through the activation of CaMKII and/or PKA. Ca^{2+} alone was able to decrease $[^3H]$-CNQX binding over a concentration range of 0.1 to 1 mM, an effect which could be blocked by specific inhibitors of CaMKII or PKA.

These data indicate that AMPA and veratridine, agents intended to mimic aspects of synaptic transmission, lead to the regulation of AMPA receptors via a Ca^{2+} influx through voltage-gated Ca^{2+} channels and the activation of specific phosphorylating enzymes. These results provide for a novel mechanism of AMPA receptor regulation and may establish the framework for a clearer understanding of the modulation of synaptic activity in normal conditions, following modifications of synaptic strength, and in some forms of neuropathology.

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AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
AP5	DL-2-amino-5-phosphonovaleric acid
ßark	ß-adrenergic kinase
BOAA	ß-N-Oxalylamino-L-alanine
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
Cl^-	Chloride
CNQX	$[^3H]$-6-cyano-7-nitroquinoxaline-2,3-dione
cAMP	Cyclic 3',5'-adenosine monophosphate
2-DG	2-deoxy-D-glucose
Dul^+	Dulbecco's$^+$
$GABA_A$	γ-amino-butyric acid$_A$
G-Protein	Guanyl-nucleotide-binding protein
IP	Inhibiting peptide
K^+	Potassium
KN-62	1-[N,O-bis(1,5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine
LTD	Long-term depression
LTP	Long-term potentiation
mACh	Muscarinic acetylcholine
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
Na^+	Sodium

nACH	Nicotinic acetylcholine
NBQX	6-nitro-7-sulfamoylbenzo-(f)quinoxalin-2,3-dione
NMDA	N-methyl-D-aspartate
NMS	$[^3H]$-N-methyl scopolamine
PKA	Protein kinase A
PKC	Protein kinase C
QNB	Quinuclidinyl benzilate
Rp-cAMPS	Rp-adenosine 3',5'-cyclic monophosphothioate
TBST	$[^{35}S]$-butyl bicyclophosphorothionate
TPA	12-O-tetradecanoylphorbol 13-acetate
TTX	Tetrodotoxin
v+g	veratridine + glutamate
TCP	N-(1(2-thienyl)cyclohexyl)-3,4-piperidine

GENERAL INTRODUCTION

Overview

Neocortex: Structure and Function

The cerebral cortex forms the layer of gray matter which covers the entire surface of the cerebral hemispheres in mammals. It is characterized by a laminar organization of its cellular components. In humans, the cortex has an area of approximately 1 square meter, makes up two-thirds of the neuronal brain mass, and contains approximately three-quarters of all brain synapses (Carpenter, 1988; Rakic, 1988).

Cortical neurons develop from segments of the telencephalic vesicle. Cells in the germinal zone which surround the lumen migrate peripherally to form the cortical sheath. Early during fetal development, cortical neurons begin to form six characteristic horizontal layers. All cells formed simultaneously migrate to the same cortical lamina; cells migrating later pass through deep layers to form the more superficial laminae. In primates and humans, the first cortical neurons are generated early during embryonic development with the full complement of cortical neurons being reached during the first half of gestation (Carpenter, 1988; Rakic, 1988).

The highest level of information processing in the brain occurs in the cortex. In mammals, three basic types of neocortex can be distinguished: sensory, motor, and association. Sensory cortex (visual, auditory or somatosensory) receives direct sensory input from specific thalamic nuclei. It receives messages from the

sense organs as well as messages of touch and temperature from throughout the body. Motor cortex receives indirect input from cerebellum, dorsal column nuclei, and other cortical regions and is involved in the control and coordination of motor output. Association cortex, in contrast to sensory cortex, mainly receives a variety of input from other cortical areas. Association cortex becomes dominant in amount to sensory cortex in primates and humans and appears to be responsible for the majority of cognitive function (Carpenter, 1988).

A key feature of cortical neurons is their ability to alter their response characteristics to changing input conditions. Such activity-dependent modifications can be of many forms, including long-term potentiation (LTP) (Kirkwood et al., 1993) and long-term depression (LTD) (reviewed by Linden, 1994) as well as modifications in sensory experience (Wiesel and Hubel, 1963). Such effects are often referred to as examples of 'neuroplasticity' and are thought to be qualitatively similar to those processes underlying learning and memory. While cortex is not unique in expressing neuroplasticity, the variety of neuroplastic phenomena in cortex appears to be greater than for any other brain region.

Synaptic Transmission

In the brain, neuron-to-neuron communication occurs at chemical or electrical synapses. Chemical synapses, representing the bulk of synapses in the brain, involve the release of a chemical mediator (neurotransmitter) from the presynaptic neuron and initiate current flow in the postsynaptic cell (Eccles, 1976).

The initial stage in neurotransmission begins with a presynaptic action potential reaching the axon terminal. Depolarization of the terminal is followed by an increase in calcium (Ca^{2+}) influx via voltage-gated Ca^{2+} channels leading to the release of neurotransmitter by exocytosis of synaptic vesicles into the synaptic cleft. The first postsynaptic step of neurotransmission then occurs with binding of the neurotransmitter to postsynaptic receptor proteins. The binding of a neurotransmitter to its target receptor leads to the opening of specific ion channels, ionic flow across the neural membrane, and a change in the cell's membrane potential. Cation flow into the neuron gives a depolarization which may result in a postsynaptic action potential. In contrast, anion flow into the neuron leads to a hyperpolarization of the target neuron. Neurotransmitter binding to target receptors is thus the first crucial step in postsynaptic signal transduction (Edelman et al., 1987).

Receptors: The First Postsynaptic Stage

Synaptic receptors can be divided into two major classes, ionotropic and guanyl-nucleotide-binding protein (G-protein) coupled. Ionotropic receptors are ligand-gated ion channels, which, upon neurotransmitter/receptor interactions, transiently open an associated ion channel leading to an ionic current flux (reviewed by Raymond et al., 1993a). In contrast, G-protein coupled receptors can indirectly activate ion channels. For these receptors, the interaction between the receptor and the ion channel is mediated by a G-protein. Neurotransmitter binding to G-protein coupled receptors usually leads to the activation of effector enzymes that produce intracellular second messengers, such as cyclic 3',5'-adenosine monophosphate (cAMP). The second messenger, in turn, acts on the channel to modulate channel function through

the activation of enzymes such as protein kinases. In some cases, G-proteins can also interact with ion channels directly. Synaptic transmission mediated by G-protein coupled receptors is slower compared to that of ionotropic receptors and usually leads to modulatory changes by altering the threshold of cells (Edelman et al., 1987b).

Ionotropic Receptors

Ionotropic receptors (ligand-gated ion channels) are pentameric structures consisting of homologous subunits which surround a central aqueous pore. Each subunit consists of four transmembrane spanning regions labeled M1 through M4. An intracellular cytoplasmic loop located between M3 and M4 has been shown to contain phosphorylation sites for a variety of protein kinases in all ionotropic receptor subunits cloned so far. An extracellular amino terminal sequence contains the agonist binding site for the receptor (reviewed by Raymond et al., 1993a; Swope et al., 1992; Huganir and Greengard, 1990). Examples of such receptors are the nicotinic acetylcholine (nACh), γ-amino-butyric acid$_A$ (GABA$_A$), glycine, and some glutamate receptors.

The primary function of ionotropic receptors is to mediate fast synaptic transmission in the brain (reviewed by Raymond et al., 1993a). The binding of specific neurotransmitters to target ionotropic receptors on the postsynaptic membrane leads to rapid alterations in the membrane permeability to particular ions, providing the basis for neural inhibition and excitation. For example, the amino acid, GABA, is the primary inhibitory neurotransmitter in brain. When GABA binds to GABA$_A$ receptors, associated chloride (Cl$^-$) channels open and Cl$^-$ flows into the cell giving rise to a hyperpolarizing transmembrane potential

(reviewed by Stephenson, 1988). Glutamate provides the basis for rapid neural excitation in the brain (Monaghan et al., 1989). When glutamate binds to glutamatergic ionotropic receptors, it opens associated cation channels, allowing some ions to enter the cell and giving rise to a depolarizing transmembrane potential.

Ionotropic glutamate receptors can be divided into three distinct subtypes, adopting the name of their preferred agonist: i) N-methyl-D-aspartate (NMDA), ii) kainate, and iii) α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (Monaghan et al., 1989). In addition to these three ionotropic subtypes, a G-protein-coupled (metabotropic) glutamate receptor has been identified (Monaghan et al., 1989). Recent molecular cloning studies have identified homologous subunits for the NMDA (NMDA R1, NMDA R2A-D), kainate (GluR 5-7, Ka 1, KA 2), and AMPA (GluR 1-4) ionotropic receptor subtypes. NMDA receptors are characterized by a voltage-dependent magnesium (Mg^+) block as well as by a high permeability to Ca^{2+}. In contrast, kainate and AMPA receptors mainly flux Na^+ (Seeburg, 1993).

AMPA receptors have been the focus of many studies. They appear to mediate the majority of fast excitatory synaptic currents in the central nervous system (Seeburg, 1993). In addition, they seem to play key roles in several forms of neuroplasticity, including LTP (Maren et al., 1993; Tocco et al., 1992) and LTD (reviewed by Linden, 1994; Linden and Conner, 1993). The mechanism involved in the regulation of these receptors has thus become an important issue in the study of nervous system function.

Protein phosphorylation catalyzed by protein kinases has been recognized as one of the primary mechanisms for regulating the activities for a wide variety of proteins (reviewed by Edelman et al., 1987), including neurotransmitter receptors (reviewed by Swope et al., 1992; Huganir and Greengard, 1990). Phosphorylation of all ionotropic receptors identified so far has been shown to lead to the modification of receptor function (reviewed by Raymond et al., 1993a; Swope et al., 1992; Huganir and Greengard, 1990).

The recent sequencing of the AMPA receptor has allowed the identification of consensus sequences for phosphorylation by serine/threonine kinases such as Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (PKC) on the major intracellular loop of all four AMPA receptor subunits (GluR 1-4) (Boulter et al., 1990; Keinanen et al., 1990). Moreover, low affinity phosphorylation sites for protein kinase A (PKA) may be present on GluR 1-4 (Kennelly and Krebs, 1991). Since these enzymes are known to be highly expressed in the CNS (Hanson and Schulman, 1992; Nairn et al., 1985; Walaas et al., 1983 a&b), and the role of phosphorylation reactions in the regulation of receptor function has been widely documented, studies have centered on the potential roles of these enzymes in the regulation of AMPA and other ionotropic receptors.

Ionotropic Receptor Regulation: Definitions

'Regulation', 'sequestration', 'up/down-regulation' and 'desensitization' are terms which are often used to describe the functional modification of receptors. Regulation is often used to describe alterations in receptor characteristics, such as receptor number or affinity. 'Sequestration' refers to a process of

internalization of cell surface receptors leaving them unresponsive to extracellular signals. Sequestration has been suggested to play a role in the regulation of G-protein coupled receptors (for review see Hausdorff et al., 1990). The extent to which sequestration operates to regulate ionotropic receptors is not known. Receptor-mediated responses may also be diminished by the degradation of existing receptors thereby reducing functional receptor number. This is termed 'down-regulation' and may be defined as a decrease in overall receptor number from a 'receptor pool' which includes both cell surface and internal receptors (reviewed by Hausdorff et al., 1990). 'Up-regulation' refers to an increase in overall receptor number, sometimes involving synthesis of new receptor proteins. For events such as sequestration or up/down-regulation the time course is usually relatively long, with a time frame of many hours to days (Maloteaux et al., 1987; Klein et al., 1979), whereas receptor regulation often occurs within minutes to hours (Yang et al., 1994; Kitamura et al., 1993; Tabuteau et al., 1993; Lanius and Shaw, 1993; Shaw and Scarth, 1991; Shaw et al., 1989; Luqmani et al., 1979; Siman and Klein, 1979). Studies examining receptor regulation, sequestration, and up/down-regulation typically use radioligand binding assays to assess the characteristics of the neurotransmitter binding site. In the following chapters the term 'regulation' will be taken to mean any change in receptor binding characteristics, only.

In contrast to receptor regulation, receptor desensitization is a term which refers to a decrease in cellular response to agonist in the continued presence of agonist. Receptor desensitization is usually studied by employing electrophysiological techniques to measure post-synaptic receptor-evoked currents. Alterations in such currents may be due to various aspects of ion channel properties, including the probability of channel opening, the number of

active channels, as well as channel unitary conductance (Raymond et al., 1993b; Greengard et al., 1991; Wang et al., 1991). A crucial point here is that alterations in receptor-mediated currents may reflect either changes in binding at the agonist binding site and/or alterations in the associated ion channel.

In the section below, the effects of phosphorylation on receptor regulation as well as receptor-mediated currents will be discussed for AMPA and other ionotropic receptors.

Ionotropic Receptor Regulation by Phosphorylation

AMPA Receptors

Modification of AMPA receptor function by CaMKII, PKC and PKA- mediated phosphorylation has been the focus of several studies (Soderling et al., 1994; Tan et al., 1994; McGlade-McCulloh et al., 1993; Keller et al., 1992; Shaw et al., 1992b; Greengard et al., 1991). A recent study has shown that activation of CaMKII present endogenously in synaptosomes resulted in strongly enhanced phosphorylation of GluR1. Activation of PKC slightly enhanced phosphorylation of GluR1, while activation of PKA resulted in little, if any, phosphorylation of GluR1. Similar results were reported when postsynaptic densities were used as the source of glutamate receptors and endogenous protein kinases. In this preparation only CaMKII strongly enhanced phosphorylation of GluR1; PKC and PKA had no significant effect. Activation of CaMKII also led to an enhancement of AMPA receptor-mediated currents in cultured hippocampal neurons, suggesting that AMPA receptors are highly modulated by their phosphorylation state (Tan et al., 1994; McGlade-McCulloh et al., 1993).

In contrast to the above studies, PKA has been demonstrated to increase the opening frequency and the mean open time of the AMPA receptor channels in hippocampal pyramidal neurons (Greengard et al., 1991). Furthermore, Keller et al. (1992) reported that bath application of a membrane permeable analogue of cAMP, an activator of PKA, potentiated currents through AMPA receptor channels comprised of GluR1 and GluR3 subunits expressed in *Xenopus* oocytes. Other results have shown that exposure of adult cortical rat slices to the catalytic subunit of PKA resulted in a 30% decrease in $[^3H]$-6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) binding. This effect could be completely blocked using a specific peptide inhibitor of PKA (Shaw et al., 1992). Thus while the effects of PKA differed in the studies cited above, a role for PKA in AMPA receptor regulation seems warranted.

Phosphorylation of other Ionotropic Receptors

Receptor regulation by phosphorylation is not unique to the AMPA receptor population. Consensus sequences for various protein kinases have been identified for many other ionotropic receptors, including the excitatory kainate and NMDA receptors as well as the nACh, $GABA_A$, and glycine receptors (reviewed by Swope et al., 1992). In the following sections, a brief review of the role of phosphorylation in the functional modification of these receptors will be presented.

Kainate Receptors

The amino acid sequence of the high affinity kainate GluR 6 subunit reveals a strong consensus sequence for phosphorylation by PKA (Egebjerg et al., 1991).

Raymond et al. (1993b) have recently reported that the GluR 6 glutamate receptor expressed in mammalian kidney cells was directly phosphorylated by PKA, and that intracellularly applied PKA was able to increase the amplitude of the glutamate mediated response. Site specific mutagenesis of the serine residue 684 abolished PKA-mediated phosphorylation of this site and eliminated the potentiation of the glutamate evoked currents. PKA has also been shown to result in an increase in kainate mediated currents in other preparations, including retinal horizontal cells (Liman et al., 1989) and cultured hippocampal neurons (Wang et al., 1991). Furthermore, Ortega and Teichberg (1990) reported that the *in vitro* phosphorylation of a putative subunit of the chick cerebellar kainate receptor by the catalytic subunit of PKA appeared to be decreased in the presence of the agonists kainate and domoate, thus suggesting a possible interaction between agonist binding and receptor phosphorylation.

NMDA Receptors

The excitatory NMDA receptor population has also been shown to be modulated by phosphorylation. In isolated trigeminal neurons, PKC potentiated NMDA-mediated currents by reducing the voltage-dependent Mg^{2+} block of NMDA-receptor channels (Chen and Huang, 1992). These results are supported by various studies which also reported a PKC-mediated enhancement of the amplitude of NMDA-receptor mediated currents in oocytes (Kelso et al., 1992), hippocampal CA1 neurons (Aniksztejn et al., 1992), and spinal cord neurons (Gerber et al., 1989). In addition, radioligand binding studies have shown that phorbol ester-induced activation of PKC decreased the K_d but not the B_{max} of $[^3H]$-MK-801 (non-competitive NMDA receptor

antagonist) binding. In contrast, phorbol ester treatment had no effect on [^{3}H]-CGS-19755 (competitive NMDA antagonist) binding (Kitamura et al., 1993). These findings suggested that activation of PKC specifically affects the NMDA receptor channel without having an effect on the NMDA agonist binding site.

Nicotinic Acetylcholine Receptors

The nACh receptors are similar to the excitatory amino acid receptors in that their activation leads to depolarizing transmembrane potentials by increasing the membrane permeability to cations. The amino acid sequence of various nACh receptor subunits reveals consensus sequences for phosphorylation by PKA, PKC, as well as a tyrosine-specific protein kinase (reviewed by Swope et al., 1992; Huganir and Greengard, 1990). Electrophysiological studies in *Torpedo californica* have shown that phosphorylation of nACh receptors by PKA, PKC, and an unidentified protein tyrosine kinase can result in the functional modification of these receptors, leading to an increase in the rate of desensitization (Hopfield et al., 1988; Huganir et al., 1986).

GABA$_A$ Receptors

Scrutiny of the sequence of GABA$_A$ receptor subunits also revealed the existence of consensus sequences for PKA, PKC, and a protein tyrosine kinase (reviewed by Swope et al., 1992; Huganir and Greengard, 1990). Purified preparations of GABA$_A$ receptors could be phosphorylated directly by both PKC and PKA (Browning et al., 1990; Kirkness et al, 1989), as well as by an unidentified kinase present in partially purified preparations of GABA$_A$ receptors (Sweetnam et al., 1988). Physiological studies examining the effects

of PKA on GABA$_A$ receptor function have led to contradictory results. Exposure of cells to the catalytic subunit of PKA or activators of PKA has been shown to attenuate (Porter et al., 1990; Tehrani et al., 1988) or, conversely, potentiate (Kano and Konnerth, 1992) GABA$_A$ receptor-mediated currents. Recent studies have demonstrated that the attenuation of GABA$_A$ receptor-mediated currents by PKA is associated with increased phosphorylation of the GABA$_A$ receptor (Leidenheimer et al., 1991; Moss et al., 1992). Moreover, PKA-mediated phosphorylation of the GABA$_A$ receptor appeared to decrease the extent of rapid desensitization to agonist (Moss et al., 1992). Radioligand binding studies have reported a decrease in [^{3}H]-SR 95531 (competitive GABA$_A$ antagonist) binding after treatment with the catalytic subunit of PKA. This effect could be blocked by a specific PKA inhibiting peptide (Shaw et al., 1992b).

Glycine Receptors

Glycine receptors are another inhibitory receptor population which have been shown to be regulated by protein phosphorylation. Song and Huang (1990) have reported that intracellular application of cAMP to cultured trigeminal neurons leads to a 70% increase in the amplitude of glycine evoked currents. In contrast, Ruiz-Gomez et al. (1991) have shown that glycine receptors purified from rat spinal cord are phosphorylated by PKC, but not PKA on a specific serine residue. It is therefore possible that PKA was exerting its effects indirectly on glycine receptors. Vaello et al. (1992) subsequently showed that the rate of phosphorylation of the glycine receptor by PKC was higher in the presence of agonist than in the presence of antagonists. Moreover, they reported a 3-fold decrease in [^{3}H]-strychnine binding when this protein was

phosphorylated by PKC, thus providing evidence that phosphorylation can alter agonist binding.

In summary, receptor phosphorylation appears to be a common way of modulating ionotropic receptor function and is by no means unique to the AMPA receptor population. Phosphorylation by a variety of protein kinases has been shown to regulate the receptor associated ion channel as well as the agonist binding site for several receptor populations studied thus far. The functional consequences of such phosphorylation, however, can be diverse depending on the specific receptor or kinase involved.

The Role of AMPA Receptors in Neuroplasticity

The term neuroplasticity broadly refers to the ability of neurons to alter some functional property in response to alterations in input. Such alterations are often long-lasting, if not permanent, and are thought to be qualitatively similar to the processes providing the basis of learning and memory (reviewed by Bliss and Collingridge, 1993). AMPA receptors have been suggested to play a role in two important model systems of neuroplasticity, namely LTP (Maren et al., 1993; Tocco et al., 1992) and LTD (reviewed by Linden, 1994).

LTP

In the hippocampus and cortex, periods of intense electrical stimulation (tetani) (usually a train of 50-100 stimuli at 100 Hz or more) in specific neural circuits result in an enhancement of synaptic transmission. This phenomenon, referred to as LTP, can persist for many hours in an *in vitro* slice preparation or in the

anaesthetized animal, and for days when induced in the freely moving animal. LTP has been studied most extensively in pyramidal cells of the CA1 region in hippocampus following stimulation of the Schaffer collateral pathway. However, it can also be elicited in area CA3 of the hippocampus, in the neocortex, as well as in various other areas in the central nervous system (see Bliss and Collingridge, 1993 for review).

NMDA receptor activation and subsequent influx of Ca^{2+} has often been shown to be necessary for the induction of some forms of LTP (for review see Bliss and Collingridge, 1993). However, increasing evidence points to a crucial role of AMPA receptors in the maintenance of LTP. Tocco et al. (1992) have reported an increase in [^{3}H]-AMPA but not [^{3}H]N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine ([^{3}H]-TCP) (non-competitive NMDA receptor antagonist) binding in hippocampal sections of animals that exhibited LTP after stimulation of the perforant pathway. This increase in binding could be blocked by administration of NMDA receptor antagonists prior to tetanic stimulation. Further studies by Maren et al. (1993) showed an increase in [^{3}H]-AMPA binding in the dentate gyrus that was highly correlated with the magnitude of LTP recorded in this structure. These changes in [^{3}H]-AMPA receptor binding were attributable to changes in AMPA receptor number rather than affinity as determined by saturation binding analyses.

Studies have also shown a gradual increase in sensitivity to AMPA following the induction of LTP. This effect, however, took at least one hour to reach its maximum and could be blocked by DL-2-amino-5-phosphonovaleric acid (AP5), a NMDA antagonist, as well as by K-252b, a potent PKC inhibitor. These latter results together with the increase in [^{3}H]-AMPA binding following NMDA

receptor-dependent LTP described above suggest that a NMDA-dependent modification of AMPA receptors may be responsible for the maintenance of some forms of LTP (Reymann et al., 1990).

Recent reports have suggested that AMPA receptors can exist in one of two forms termed 'flip' and 'flop'. The 'flip' form produces a higher conductance channel and desensitizes less rapidly than the 'flop' form (Sommer et al., 1990). Collingridge and Singer (1991) have speculated that the activation of kinases via NMDA receptor activation during the induction of LTP may result in an increase in the 'flip' form of AMPA receptors, leading to an increase in synaptic efficacy through higher conductance, less rapidly desensitizing AMPA receptors.

LTD

LTD is a form of synaptic plasticity in which prolonged periods of low-frequency stimulation (usually 1-3 Hz) elicit a synaptic depression that is input-specific and persists for many hours. Although this phenomenon has not been studied in chronic preparations, studies using acute intact animals or cell cultures have shown the synaptic depression to persist for many hours (reviewed by Linden, 1994). LTD in cerebellum appears to manifest itself as a decrease in AMPA-mediated currents as a result of protein kinase activity (Linden et al., 1993, 1991; Ito and Karachot, 1992). Several processes are necessary and sufficient for the induction of cerebellar LTD: Na^+ influx through AMPA receptor ion channels, Ca^{2+} influx via voltage-gated Ca^{2+} channels, and metabotropic glutamate receptor activation and subsequent activation of PKC leading to alterations in AMPA receptor function (Linden, 1994; Linden et al., 1993; 1991).

Although Na$^+$ influx through voltage-gated Na$^+$ channels is sufficient to induce LTD in some cases, activation of AMPA receptors appears to be much more effective (Linden et al., 1993). In contrast to the induction of LTD, there is strong evidence that the expression of this phenomenon is mediated entirely by alterations in the number or sensitivity of postsynaptic AMPA receptors as a result of protein kinase C activity (Linden, 1994; Linden et al., 1993; 1991). Ito and Karachot (1992) further reported that activation of PKC by phorbol esters could mimic LTD, thus suggesting that LTD may result from phosphorylation and concomitant regulation of AMPA receptors by PKC.

It is interesting to note that the expression of cerebellar LTD is thought to be mediated by the modulation of AMPA receptors as a result of PKC activation. McGlade-McCulloh et al. (1993) did not report a change in the AMPA receptor-mediated currents in rat hippocampal pyramidal cells by PKC. These differences in the action of PKC, however, may be explained by differences in GluR subunits among the different brain regions studied. Cerebellar Purkinje cells are known not to possess GluR1 and GluR4, whereas both of these subunits are known to be present in cortex and hippocampus (Martin et al., 1993). In support of this view, it has been reported that receptor phosphorylation has differential effects depending on the subunit composition of the receptor (Krishek et al., 1994; Moss et al., 1992).

In summary, AMPA receptors clearly play a significant role in some forms of neuroplasticity such as LTP and LTD. Protein kinase-mediated modulation of the AMPA receptor population has been suggested to occur after the induction of both LTP and LTD and may be involved in the maintenance of both of these phenomena.

The Role of AMPA Receptors in Neurodegenerative Diseases

Neuronal cell death may be caused by increased intracellular Ca^{2+} as a result of an overstimulation of excitatory amino acid receptors by glutamate or aspartate (reviewed by Lipton and Rosenberg, 1994). Such 'excitotoxic' actions have been reported to contribute significantly to neuronal loss occurring after acute and chronic forms of neuronal degeneration (Meldrum and Garthwaite, 1991). Ischemia, an acute form of neural degeneration, has been shown to be associated with AMPA receptor-mediated excitotoxicity. The main evidence for this comes from pharmacological studies which showed that the competitive AMPA receptor antagonist 6-nitro-7-sulfamoylbenzo(f)quinoxalin-2,3-dione (NBQX) appeared to protect against cortical damage resulting from complete global ischemia even when there was significant post-ischemic delay before drug administration (Sheardown et al., 1990). These latter findings suggested an important role for AMPA receptors in the mechanisms underlying ischemia and suggested that overstimulation of AMPA receptors may have effects on other processes leading to neuronal cell death. For example, AMPA receptors have been shown to play a key role in neurolathyrism, a chronic spastic neurodegenerative syndrome found predominantly in East Africa and Southern Asia. Neurolathyrism is associated with dietary consumption of the chick pea *Lathyrus sativus,* a plant containing the toxin ß-N-Oxalylamino-L-alanine (BOAA). BOAA acts as an AMPA receptor agonist (Bridges et al., 1989) on spinal cord neurons and eventually leads to selective destruction of upper and lower motor neurons (Meldrum and Garthwaite, 1991). Although it remains to be determined why a toxin given systemically leads to the lesioning of only very selective neuronal populations, a possible explanation is that only AMPA

receptors present in upper and lower motor neurons contain binding sites for this particular toxin.

Thesis Objectives

The literature clearly reveals important roles for AMPA receptors in normal excitatory neural transmission in the central nervous system and links these receptors to some forms of neuroplasticity and neuropathology. A clearer understanding of the molecular mechanism involved in activity-dependent regulation of AMPA receptors may provide new insights into these events. Studies of the characteristics and regulation of these receptors are thus important goals and the major focus of this thesis.

Using radioligand binding assays employing the competitive AMPA receptor antagonist $[^3H]$-6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), activity-dependent AMPA receptor regulation in rat neocortex will be examined. Chapter 1 will describe characterization studies of a $[^3H]$-CNQX binding site. Chapter 2 will use $[^3H]$-CNQX binding to examine the regulation of the AMPA receptor population to agonist and depolarizing stimuli, stimuli which are believed to mimic aspects of synaptic neurotransmission seen *in vivo*. Chapter 3 will examine the effects of phosphorylating enzymes (i.e. protein kinases) on $[^3H]$-CNQX binding. Chapter 4 will establish that agonist and depolarizing stimuli exert their effects through the activation of particular protein kinases. Finally, Chapter 5 will examine the role of specific ionic species in the cascade of events leading to kinase-mediated regulation of the AMPA receptor agonist binding site.

GENERAL METHODS

The Cortical Slice as a Model System

Models are simplified representations of biological processes. They are used in situations when it is not possible to study the phenomenon of interest *in vivo,* as is often the case in the biological sciences (Zbinden, 1992). At a neural systems level, a model preparation might employ an isolated neural circuit rather than the whole structure, e.g. the study of LTP in cortical or hippocampal slices rather than in the intact brain. In the latter case, a large number of variables remain uncontrolled. Such variables include the concentration of administered drugs, temperature, as well as synaptic connections with other brain structures. Many such variables, however, can be better controlled in slice or other isolated preparations. The work described in this thesis uses a cortical slice preparation in which most cells are alive and in relatively intact neural circuits in order to study activity-dependent AMPA receptor regulation.

"Living" Slice Preparation: Advantages and Disadvantages

The study of neurotransmitter receptors in the central nervous system has often employed techniques in which the cells are completely or partially disrupted. Examples of such techniques include the widely used homogenate preparations (for review see Bylund and Yamamura, 1990; Yamamura et al., 1990) or those employing thin *in vitro* sections (for review see Young and Kuhar, 1987; Unnerstall et al., 1982). Although these techniques have provided substantial information about receptor characteristics as well as the regional distribution of many receptors, they are limited in terms of studies of receptor

function in <u>living</u> (intact) cells under physiological conditions (for review see Shaw and Wilkinson, 1994; Wilkinson et al., 1986). A "living" tissue preparation therefore offers the following advantages over homogenate or *in vitro* autoradiographic techniques:

(1) Since the receptors are on living cells which are still in relatively intact neural circuits, assays can be performed under conditions which are as close to physiological conditions as possible. Neurons are thus affected by processes which occur in living cells such as depolarization and neurotransmitter release. (2) Homogenate and *in vitro* autoradiographic techniques may conceal the effects of experimentally-induced receptor regulation. For example, an experimental paradigm that may lead to the sequestration/internalization of receptors as part of the regulation process may fail to reveal changes in receptor binding because of the inability to distinguish internal from membrane bound receptors. In a "living" slice preparation in which the majority of plasma membranes are intact, however, membrane and internal receptors can be distinguished. This is usually accomplished by the use of hydrophilic versus lipophilic radioligands. Due to the inability of hydrophilic ligands to cross plasma membranes, these radioligands label membrane bound receptors only. Lipophilic radioligands, on the other hand, are able to cross plasma membranes and therefore label both membrane and internal receptors. (3) The use of relatively intact tissue may diminish the release of proteolytic enzymes for receptors and/or neurotransmitters whose activity might lower binding levels.

Although the above mentioned points are distinct advantages of the "living" slice technique, there are also several disadvantages to using this technique. (1) Receptor regulation studies using radioligand binding cannot determine on

which cell types receptor regulation is occurring. It is therefore impossible to distinguish neuronal from glial cell receptor regulation using this preparation. (2) Naturally occurring agonists, e.g. glutamate (see Chapters 1 and 2; Lanius and Shaw, 1992), GABA (Shaw and Scarth, 1991), and ACh (Van Huizen et al., 1989) often fail to show an effect in both competition and regulation experiments. This may, in part, be due to the presence of functional uptake sites as well as degradative enzymes which are still present in intact tissue (for review see Shaw and Wilkinson, 1994). It is therefore difficult to examine the roles of putative neurotransmitters in receptor regulation using a "living" slice preparation. However, this problem can be overcome by employing agonist analogues. (3) Only radioligands whose dissociation rate constants are slower than the time required to rinse out the free ligand can be used for radioligand binding in intact slices. It is for that reason that antagonists generally appear to work better than agonists.

Animals

All experiments were performed with adult male Sprague-Dawley rats (>60 days). The rats were raised under controlled lighting conditions (12:12 h light-dark cycle) and had free access to food and water.

Preparation of Brain Slices

Animals were anaesthetized with halothane and sacrificed by decapitation. The whole brain was then removed in less than 1 minute from the time of decapitation and immersed in a modified ice-cold Dulbecco's phosphate buffered saline solution (Gibco, Grand Island, NY). The modified medium ,

'Dulbecco's plus' (Dul+), contained glucose (1 mg/mL), Hepes (25 mM), and hydrogen peroxide (0.003%), the latter added as a source of molecular oxygen (Walton and Fulton, 1983).

After removal of the brain, blocks of cortex approximately 5 mm in length by 2mm in width were rapidly dissected out and placed in cold Dul+ (4°C). The blocks contained mainly sensory areas including parietal cortex areas 1,2,3 and 7 and occipital cortex area 17 and 18 (Krieg, 1946) as well as some adhering white matter. Coronal tissue slices of approximately 400 μm thickness were obtained with a tissue slicer (Bennett et al., 1983). The 25 slices that were normally obtained from each rat cortex were separated and placed at random in the wells of tissue culture plates, each well containing 0.5 mL of cold Dul+. Random placement of slices in the wells allowed for the control of variations in slice size.

Evidence for Slice Viability

The brain slice preparation described above is essentially identical to the brain slice preparation used for electrophysiological studies by many laboratories (Reid et al., 1988; Teyler, 1980; McIlwain et al., 1951). Cortical slices are thought to contain mostly living cells for the following reasons:

(1) Trypan blue, a dye that cannot be taken up by cells with intact plasma membranes (Tennant, 1964), labeled many cells at the cut edges of the slice, but relatively few towards the center of the slice. Quantification of the percentage of dead cells was estimated to be approximately 15% at 0 h and 30% after 6 h at 30°C (Van Huizen et el., 1989). It is worth noting that some

cells in the interior of the slice were labeled showing that the dye has access to all areas of the slice.

(2) Electron microscopic examination revealed that cells at the edges of the slice appeared to be mostly dead cells, with the center of the slice containing healthy tissue for up to 6 h at 30°C (Van Huizen et al., 1989). At the edges of the slice, a large number of swollen dendrites without their cytoplasmic organelles were seen. However, dendrites in the center of the slice still contained cytoplasmic organelles, their membranes were intact, and axon terminals were making synapses on somata as well as on dendritic spines (Van Huizen et al., 1989).

(3) Electrophysiological experiments showed that field action potentials could be obtained in layers 2/3 of visual cortex after stimulation of the optic radiation (Van Huizen et al., 1989).

(4) Cells showed 2-deoxy-D-[^{14}C]glucose uptake, a marker of cellular viability (Sokoloff et al., 1977), for up to 6 hours (see Chapter 2 and Van Huizen et al., 1989).

Radioligand Methods

A variety of techniques can be used to study neurotransmitter/receptor interactions. Some of these are indirect, e.g. electrophysiological methods, in that they do not allow the measurement of receptor density. Direct measurements include fluorescent- and radioligand techniques. Of the latter,

radioligand binding is a well established and quantitative method for studying receptor characteristics, regulation, and distributions.

Radioligand

$[^3H]$-CNQX (specific activity 15.6-26.7 Ci/mmol), a competitive AMPA receptor antagonist, was purchased from New England Nuclear (NEN). Although CNQX has been shown to bind to both AMPA and kainate receptors (Young and Fagg, 1991), CNQX has been shown to bind to AMPA receptors with approximately five times greater affinity (Honore et al., 1988). In the present preparation, kainate did not compete for 5-10 nM $[^3H]$-CNQX, the concentration range employed in the experiments described below (see Chapter 1 and Lanius and Shaw, 1992). The use of 5-10 nM concentrations of $[^3H]$-CNQX should therefore provide a relatively specific measure of AMPA receptor binding.

The choice of a competitive antagonist (CNQX) instead of an agonist (AMPA) is often preferable in binding experiments in intact cells. Usually, competitive antagonists have slower dissociation rate constants than agonists. This factor is critical for radioligand binding experiments in slices because the rinse-out time for free radioligand must be shorter than the dissociation rate constant.

$[^3H]$-CNQX Binding Assays

Immediately prior to the experiment, $[^3H]$-CNQX was diluted to the desired concentration (5-10 nM) in Dul$^+$. Five hundred μL of this solution were added to the slices after the original Dul$^+$ was removed. Incubation with $[^3H]$-CNQX was allowed to proceed for 3 h (see Chapter 1 Results). Three slices were normally

used to determine total (receptor and non-receptor) binding, while two slices were used for a determination of non-specific (non-receptor) binding. Non-specific binding was determined by the addition of 5 μL of 10^{-2} M unlabeled CNQX as competitor to give a final bath concentration of 10^{-4} M. Protein content in these slices was determined by a modification of the method of Lowry et al. (1951), and specific binding could thus be expressed as femtomoles bound per mg protein.

Following the incubation step with [^{3}H]-CNQX, a 20 μL sample of the buffer in the well was removed to determine 'free' ligand concentration. The rest of the buffer was removed with a Pasteur pipette and the slices were rinsed twice for 5 min with 0.5 mL DuI$^+$ at 4°C (see Chapter 1 Results). After the final rinse, the buffer was removed, the slices were picked up with a small circle of glass microfibre filter paper (Whatman GF/B), and were placed directly in scintillation counting vials containing 4 mL of Formula 963 counting cocktail (NEN). The amount of bound ligand was determined in a LS6000 IC Beckman scintillation counter (efficiency for ^{3}H, approximately 55%) after a minimum interval of 12 h.

Statistical Analysis

Statistical analysis of binding was performed using a Student's t-test (two-tailed) or a one-way analysis of variance (ANOVA) ($p<0.05$).

CHAPTER 1

CHARACTERIZATION OF A [^{3}H]-CNQX BINDING SITE

Introduction

In order for a binding site to be identified as a receptor a number of criteria must be fulfilled. The binding site must show (1) saturability, (2) competition by appropriate pharmacological agents, (3) steady-state binding, as well as (4) the appropriate distribution (Boulton et al., 1985). A bare minimum requirement for binding is that it be saturable. Saturation of specific binding should occur at, or below, concentrations of ligand comparable to those required to produce a biological effect (typically in the nanomolar or micromolar range) (Burt, 1980). Second, pharmacological specificity is of vital importance in recognizing a binding site as a receptor. The binding of a ligand should be displaceable by compounds, including agonists and antagonists, which have been shown to mimic or inhibit the biological response of the ligand (Burt, 1980). Moreover, the ability of drugs to compete for the radioactively labeled ligand should correlate with the potencies of these drugs as either agonists or antagonists in biological systems (Burt, 1980). Moreover, the binding site must show steady-state binding after a certain time period, depending on the receptor (Boulton et al., 1985). Finally, the distribution of binding sites should be present in tissues previously demonstrated to possess the neurotransmitter of the binding site in question (Boulton et al., 1985), and the relative distribution of receptors should roughly correlate with the density of synaptic junctions utilizing that neurotransmitter (Boulton et al., 1985).

In addition to the above discussed criteria, it is imperative to show that the activation of a binding site is associated with a physiological response such as a change in membrane potential or stimulation of second messenger activity. If all the above criteria have been fulfilled, one may assume that the binding sites in question represent actual neurotransmitter receptors.

In the following chapter, characterization studies, including saturation binding, competition, and steady-state binding of a [^{3}H]-CNQX binding site in rat neocortex will be described. These experiments will test the hypothesis that [^{3}H]-CNQX is binding to an AMPA receptor population.

Materials and Methods

Brain slices were prepared as described in the General Methods.

All [^{3}H]-CNQX characterization experiments were performed at 4°C to prevent receptor regulation which may confound the results (Van Huizen et al., 1989) and to decrease binding to potential uptake sites (Liron et al., 1988). Characterization experiments included association/ dissociation time courses, competition, as well as saturation binding experiments. Time course experiments allow the calculation of the association and dissociation rate constants. The equilibrium dissociation constant (K_d) can be determined from time course experiments in which the association (k_{+1}) and dissociation (k_{-1}) rate constants are calculated. The K_d is the ratio k_{-1}/k_{+1} and should, within experimental error, be similar to the K_d determined from saturation binding experiments (Boulton et al., 1985). The dissociation rate constant, k_{-1}, can be calculated by multiplying the slope of log [B] (specific binding) against time by e

(-2.303) (Bennet, 1978). The association rate constant, k_{+1}, can be calculated from the amount of the bound ligand at various time intervals (B_t) until equilibrium (B_{eq}) has been reached from a plot of ln (B_{eq} - B_t) versus time. The slope of this line (k_{obs}) relates to both the k_{+1} and k_{-1} and free ligand concentration (L) in the following way:

$$k_{+1} = \frac{k_{obs} - k_{-1}}{[L]}$$

Competition studies used the following compounds at a concentration range from 10^{-3} M to 10^{-12} M (see Table 1 below for a description of these substances): glutamate, AMPA, CNQX, NBQX, quisqualate, NMDA and kainate. These studies allow the calculation of IC_{50} values, the concentration of unlabeled ligand required to inhibit 50% of the radioactive ligand binding (Boulton et al., 1985), and show if [^{3}H]-CNQX is indeed binding to a specific AMPA receptor.

Compound	Site of Action
glutamate	natural agonist interacts with AMPA, kainate, NMDA, and metabotropic glutamate receptors
AMPA	potent agonist which specifically interacts with AMPA receptors
CNQX	competitive AMPA/kainate antagonist (5 times higher affinity for AMPA than kainate receptors (Honore et al., 1988)) weak inhibitor of NMDA receptors because of its ability to compete with glycine for its binding site
NBQX	competitive AMPA/kainate antagonist, little affinity for the NMDA glycine site
quisqualate	AMPA/kainate and metabotropic receptor agonist
NMDA	NMDA receptor agonist
kainate	kainate/AMPA receptor agonist

Table 1: Pharmacological agents used to analyze [^{3}H]-CNQX binding to AMPA receptors in rat cortical slices (see Young and Fagg, 1991).

For saturation binding experiments a concentration range of 2 to 909 nM [^{3}H]-CNQX was used. From such experiments the dissociation equilibrium constant,

K_d, as well as the maximum number of binding sites, B_{max}, can be calculated. B_{max} and K_d values in this case were computed using nonlinear regression analyses (GraphPad PRISM programme).

In experiments designed to examine the distribution of surface versus possible internal AMPA receptors, some slices were slowly frozen to -30°C and then thawed in order to disrupt the cellular membranes prior to attempting the binding assays. A consideration of the structure of CNQX suggests that it is polar and should, therefore, have restricted passage through intact plasma membranes. The use of frozen/thawed versus intact cells labeled with [^{3}H]-CNQX should thus allow experiments to distinguish the percentage of AMPA receptors present in the plasma membrane and in intracellular compartments. In addition, to examine possible [^{3}H]-CNQX binding to uptake sites control slices incubated in Dul$^+$ were compared to those incubated in 50mM Tris-acetate, a Na$^+$ free buffer. Since uptake sites are temperature as well as sodium dependent (Kanai et al., 1993), assays performed at 4°C in Tris-acetate should eliminate the binding to potential glutamate uptake sites.

Results

To determine whether the [^{3}H]-CNQX binding site shows steady-state binding, time course experiments examining the association and dissociation of specific [^{3}H]-CNQX binding were carried out (Figure 1). At 4°C, [^{3}H]-CNQX binding reached steady-state by 150 min, remaining stable for up to 5 h. Dissociation of specific [^{3}H]-CNQX binding was determined once steady-state binding had been reached at 150 min. [^{3}H]-CNQX did not easily dissociate at 4°C, showing

approximately 40% decreases in binding after 40 min rinse times. From these experiments a 3 h incubation time for [^{3}H]-CNQX was chosen. Two 5 min rinses were adequate to achieve maximum specific binding and reduce free ligand concentration.

To evaluate the specificity of [^{3}H]-CNQX binding, the ability of various compounds to compete for [^{3}H]-CNQX binding was examined (Figures 2A & B). AMPA, CNQX and NBQX were the most effective compounds showing IC$_{50}$ values near 10^{-6} M and final non-specific binding levels of about 30%. Quisqualate was less effective having an IC$_{50}$ value near 10^{-5} M and final non-specific binding levels of approximately 58% (Figure 2A). In Fig. 2B, competition curves for the agonists glutamate, kainate and NMDA are shown. Neither glutamate, kainate, nor NMDA were effective in competing for [^{3}H]-CNQX binding.

To determine whether [^{3}H]-CNQX binding is saturable, saturation binding studies using a concentration range of 2 to 909 nM [^{3}H]-CNQX were carried out (Figure 3). [^{3}H]-CNQX labeled binding sites in a saturable manner. Nonlinear regression analyses of saturation binding data indicated that [^{3}H]-CNQX bound to a single population of receptor sites with a K$_d$=565 and a B$_{max}$=11.4 pmol/mg protein. Non-specific binding ranged from 36 % of total binding to 77 % of total binding at 2 and 909 nM [^{3}H]-CNQX, respectively.

Figure 4 illustrates [^{3}H]-CNQX binding for slices incubated under different conditions. These conditions included slices in DuI$^+$, slices in DuI$^+$ following freeze/thaw treatment, and slices incubated in 50 mM Tris-acetate buffer. These data show that the level of binding was not significantly altered from control

values at any of the tested concentrations of ligand for either the freeze/thaw or Tris-acetate treated slices (p>0.05, Student's t-test) (see Discussion).
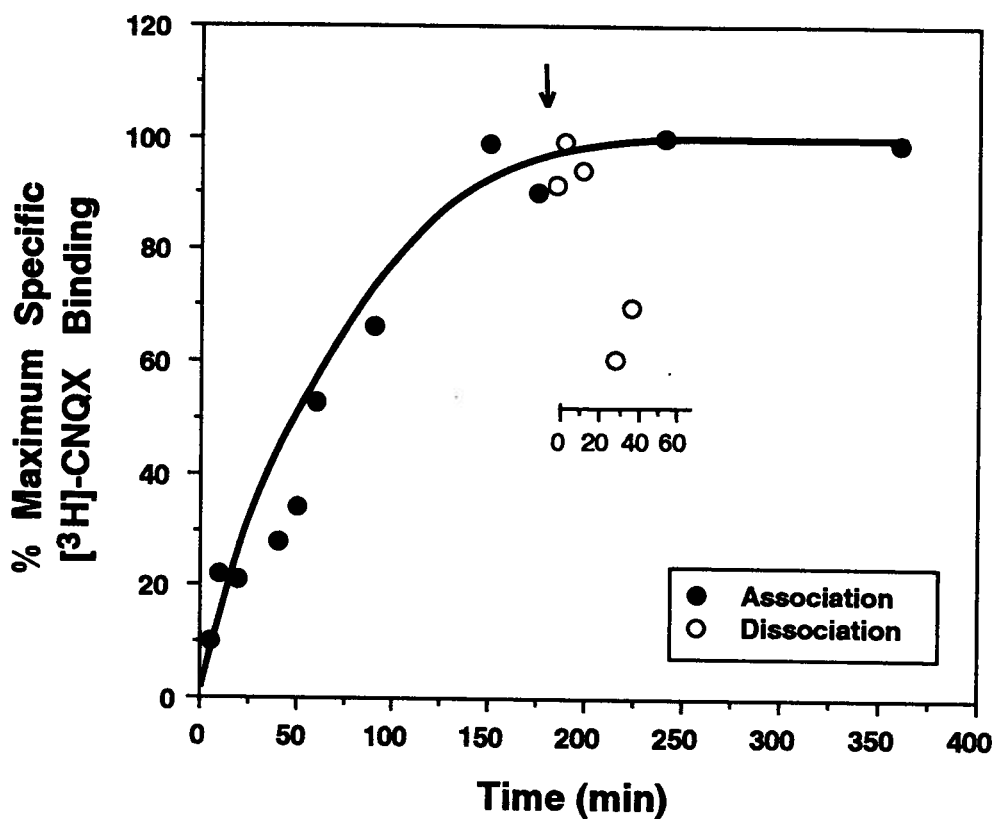
Figure 1: Association and dissociation measurements of [^{3}H]-CNQX binding in adult rat neocortical slices at 4°C. Ligand concentration was 5-10 nM. Specific binding is plotted against time for increasing incubation times (●) and a fixed rinse time (2x5 min) or for a fixed incubation time (3h) and variable rinse times (o). Rinse onset is indicated by the arrow and is shown in the inset time scale.

Figure 2: Competition of [^{3}H]-CNQX binding with specific AMPA analogues. A: competition with the AMPA agonists AMPA and quisqualate as well as with the AMPA antagonists CNQX and NBQX. B: competition with glutamate, NMDA, and kainate. In all experiments, the ligand concentration was 5-10 nM. All incubations with [^{3}H]-CNQX lasted 3 h at 4°C. Rinses were 2x5 min.

A

B
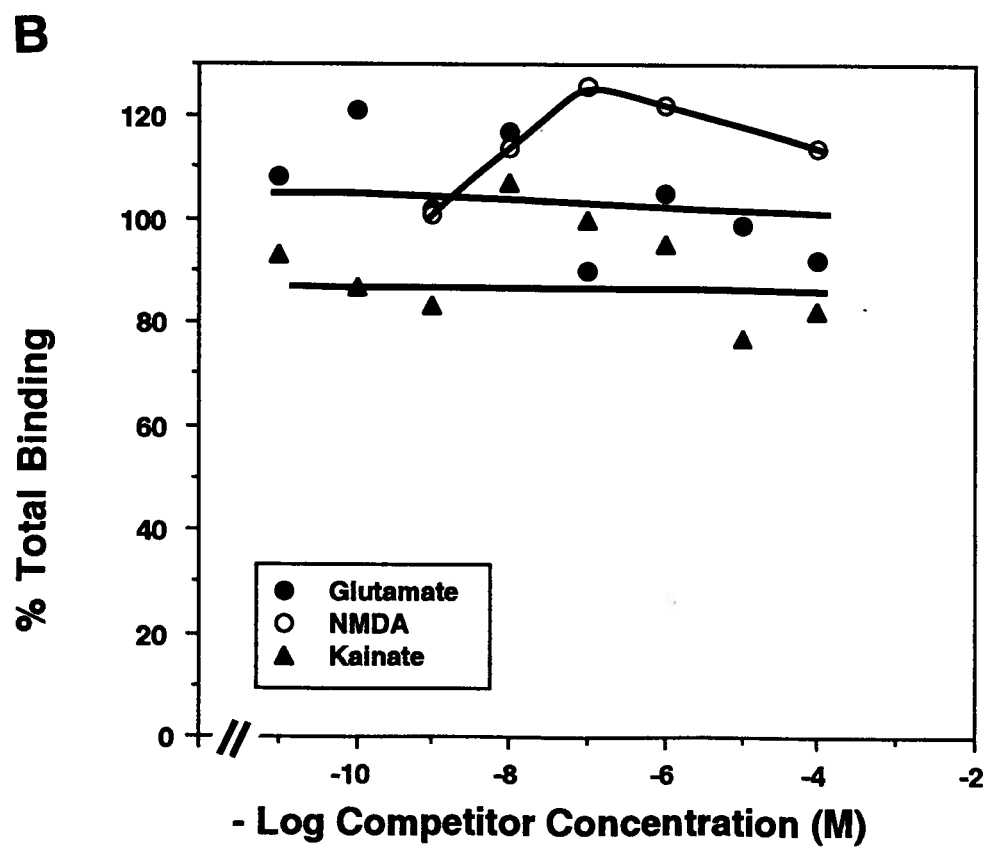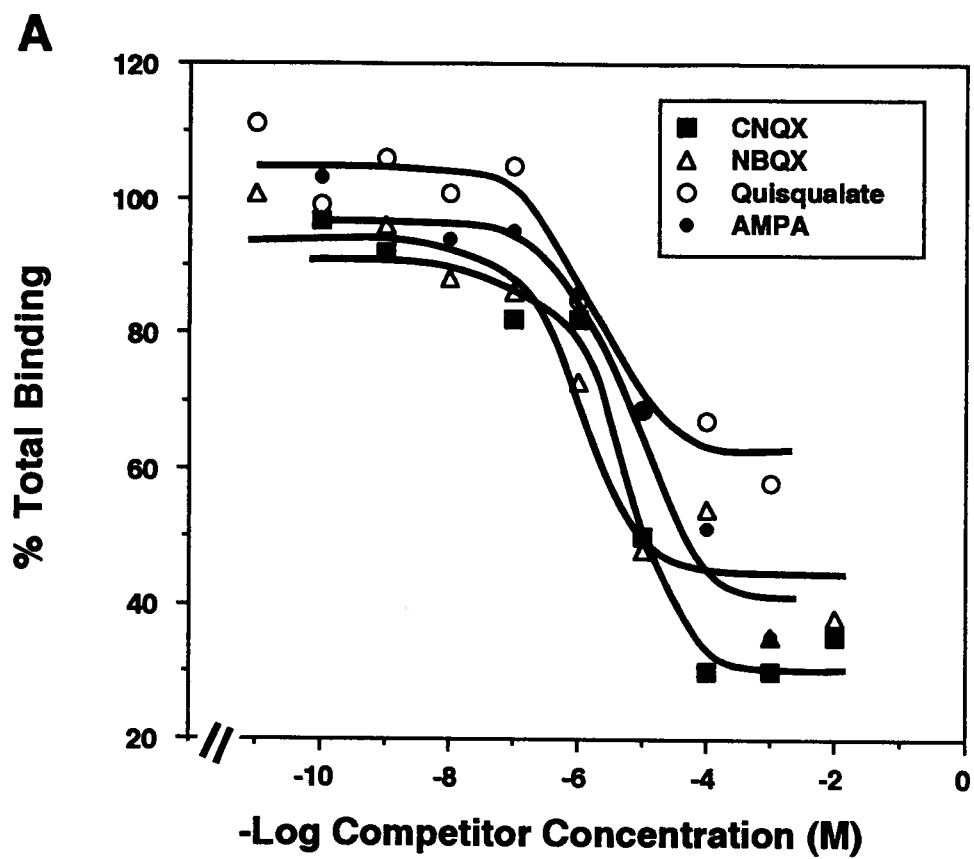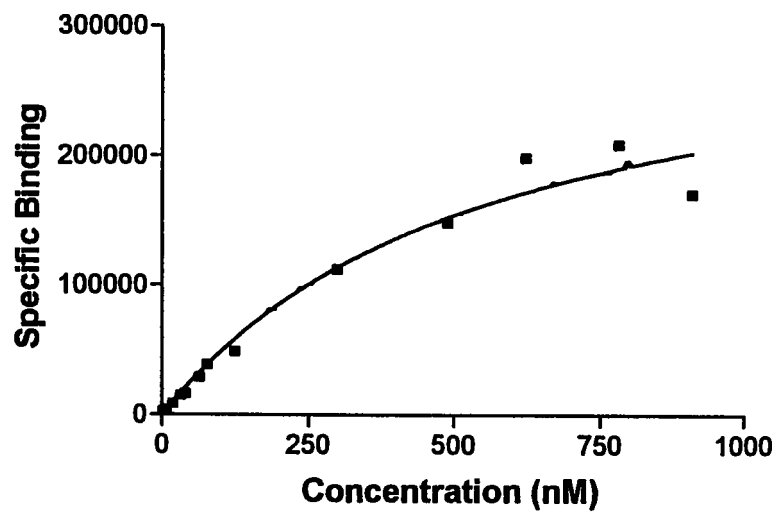
Figure 3: Saturation binding isotherm for specific [³H]-CNQX binding. The incubation time in varying concentrations of [³H]-CNQX (2-909 nM) was 3 h at 4°C. Rinse time was 2x5 min. Specific binding is expressed as dpm.
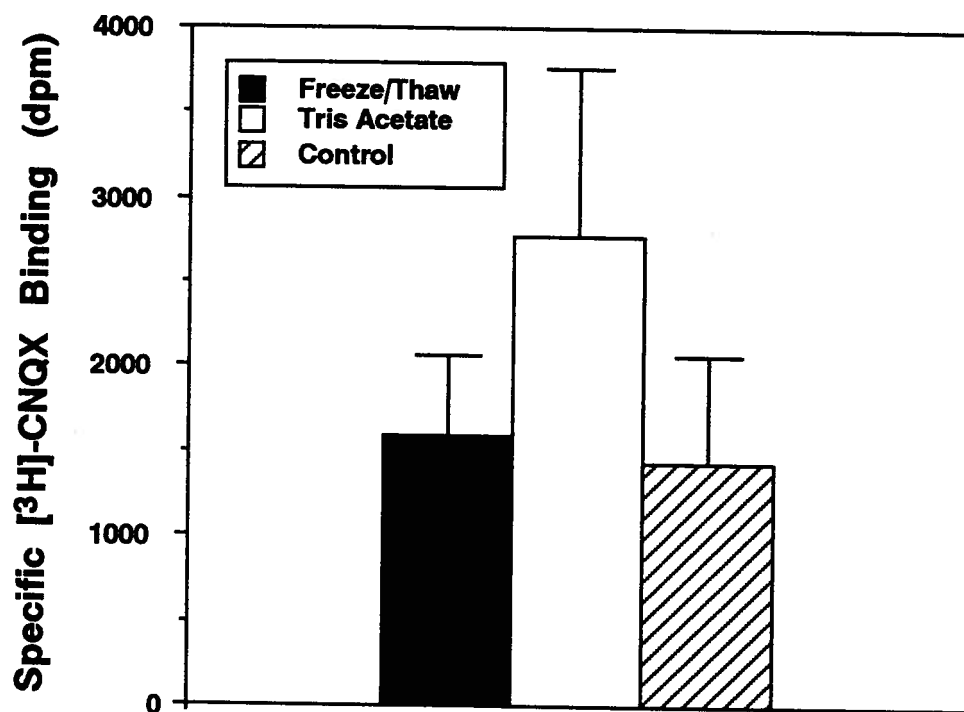
Figure 4: Effects of freeze/thaw treatment or incubation with 50 mM Tris-acetate on [³H]-CNQX binding. Ligand concentration was 5-10 nM. All incubations with [³H]-CNQX lasted 3 h at 4°C. Rinses were 2 x 5 min. Error bars give S.E.M. (n=3).

Discussion

Using *in vitro* living rat cortical slices, a [^{3}H]-CNQX binding site was characterized. Time course experiments showed that 5-10 nM [^{3}H]-CNQX reached steady-state after 150 minutes at 4°C and remained stable for up to 5 hours. Nielsen et al. (1990) reported steady-state binding of 50 nM [^{3}H]-CNQX in 20 μm sections of rat cerebellum after 15 minutes at 2°C. The discrepancy between the time needed to reach steady-state binding may be explained by the differences in the preparations and radioligand concentrations used. The use of 400 μm compared to 20 μm thick tissue may lead to a slower rate of diffusion of the radioligand into the slice, thus resulting in an apparent increase in the association rate constant. Moreover, 50 nM [^{3}H]-CNQX labels AMPA as well as kainate receptors (Nielsen et al., 1990). It is therefore possible that these combined sites possess a different association rate constant.

In the present preparation, 5-10 nM [^{3}H]-CNQX did not easily dissociate at 4°C, showing approximately 40% decreases in binding after 40 min rinse times. In contrast, Nielsen et al. (1990) showed that 50 nM [^{3}H]-CNQX binding in 20 μm thick sections of rat cerebellum was fully reversible within 30 minutes at 2°C. Although it remains unknown why [^{3}H]-CNQX binding was not fully reversible in the present preparation, differences in the preparation may be responsible for the observed differences. Since [^{3}H]-CNQX did not easily dissociate, the dissociation rate constant (k_{-1}) and K_d could not be calculated from the time course experiments.

Competition studies showed that AMPA, CNQX, NBQX, and quisqualate were effective as competitors for 5-10 nM [^{3}H]-CNQX binding. Glutamate, kainate,

and NMDA showed no competition for 5-10 nM [^{3}H]-CNQX binding. AMPA (AMPA agonist), CNQX (AMPA/kainate antagonist), and NBQX (AMPA/kainate antagonist) all had IC_{50} values near 10^{-6} M; quisqualate (AMPA/kainate and metabotropic receptor agonist) was less effective, showing an IC_{50} value of 10^{-5} M. NMDA was not able to compete for [^{3}H]-CNQX binding. Nielsen et al. (1990) reported inhibition of 50 nM [^{3}H]-CNQX binding in the molecular layer of cerebellum by AMPA (K_i 0.015 μM), quisqualate (K_i 0.77 μM), L-glutamate (K_i 5.5 μM), and kainate (K_i 20 μM), with no competition by NMDA. Although glutamate as the natural agonist of the AMPA receptor was moderately effective in the competition of 50 nM [^{3}H]-CNQX binding using *in vitro* autoradiography (Nielsen et al., 1990), it was ineffective in the present preparation. As discussed in the General Methods, one disadvantage of the "living" slice technique is that naturally occurring agonists, e.g., glutamate (Lanius and Shaw, 1993), GABA (Shaw and Scarth, 1991) and acetylcholine (Van Huizen et al., 1989), often fail to show an effect in both competition and regulation experiments. This may, in part, be due to the presence of functional uptake sites as well as degradative enzymes which are still present in intact tissue (reviewed by Shaw and Wilkinson, 1994). It is therefore difficult to examine the roles of putative neurotransmitters in receptor regulation using the present slice preparation.

Although kainate resulted in some competition of 50 nM [^{3}H]-CNQX (Nielsen et al., 1990), it was ineffective in inhibiting 5-10 nM [^{3}H]-CNQX. Since CNQX has been reported to have a five times higher affinity for AMPA than for kainate receptors (Honore et al., 1988), the use of 50 nM versus 5-10 nM [^{3}H]-CNQX concentrations could result in increased labeling of kainate receptors by [^{3}H]-CNQX. This may explain the ability of kainate to compete for 50 nM [^{3}H]-CNQX binding but not for 5-10 nM [^{3}H]-CNQX binding. Nevertheless, the lack of effect

of kainate in the present preparation suggests that at 5-10 nM, [^{3}H]-CNQX labels an AMPA receptor to which kainate does not bind.

We have previously reported the presence of an apparent high- and low-affinity [^{3}H]-CNQX binding site using ligand concentrations ranging from 1 to 75 nM. The high-affinity [^{3}H]-CNQX binding site showed a K_d=11 nM and a B_{max}=470 fmol/mg protein and appeared to saturate between 20 and 35 nM [^{3}H]-CNQX (Lanius and Shaw, 1992). The saturation binding isotherms for these experiments were fit by eye. Subsequent nonlinear regression analyses of saturation binding isotherms obtained by using ligand concentrations from 2 to 909 nM [^{3}H]-CNQX indicated that [^{3}H]-CNQX bound to a single population of receptor sites with a K_d=565 nM and a B_{max}=11.4 pmol/mg protein. These results are in agreement with previous studies (Maren et al., 1993; Honore et al., 1989; Nielsen et al., 1990) which have reported that [^{3}H]-CNQX binds with equal affinity to two states (high and low affinity) of the AMPA receptor (Honore et al., 1989; Nielsen et al., 1990). The K_d obtained from the present data is higher than the K_d previously reported by Maren et al. (1993) (131 nM), Honore et al. (1989) (39 nM), and Nielsen et al. (1990) (67 nM). However, this discrepancy may be due to the fact that nonlinear regression analyses were used to generate the present K_d value. The K_d values obtained by Maren et al. (1993), Honore et al. (1989), and Nielsen et al. (1990) were generated using conventional Scatchard analyses (linear regression).

The B_{max} value obtained in the present preparation was found to be higher than that obtained previously in the molecular layer of cerebellum (11.4 pmol/mg protein versus 3.54 pmol/mg protein) (Nielsen et al., 1990). This disparity, however, is likely attributable to the fact that higher densities of AMPA

receptor binding have been observed in some areas of rat cortex compared to the molecular layer of cerebellum (Nielsen et al., 1990; Olsen et al., 1987). The present B_{max} value was found to be similar to the B_{max} value attained in the dentate gyrus (11.2 pmol/mg protein) (Maren et al., 1993).

The experiments comparing slices incubated in Dul^+ to those which were freeze/thawed or incubated in Tris-acetate showed no significant differences in [^{3}H]-CNQX binding among the different groups. However, standard errors in these experiments were very big and are likely due to rather large variations in slice size which were unavoidable when the technique was first employed. In the first case, freeze/thawing ruptures cellular membranes, thereby exposing potential internal AMPA receptors to [^{3}H]-CNQX which, because of its polarity, does not cross intact cellular membranes. These results may be interpreted as supporting the idea that most functional AMPA receptors are on cell surfaces. In the second case, the use of a Tris-acetate buffer at 4°C was intended to examine whether [^{3}H]-CNQX was binding to potential uptake sites. Binding to uptake sites has been reported to be Na^+ (Kanai et al., 1993) as well as temperature-dependent (Zaczek et al., 1987). [^{3}H]-CNQX binding carried out in a buffer which does not contain Na^+ ions at 4°C should therefore eliminate any potential binding to uptake sites. Since brain slices incubated in Tris-actetate did not show decreased levels of [^{3}H]-CNQX binding from slices incubated in Dul^+, the present data support the view that most [^{3}H]-CNQX binding observed is to receptors rather than to uptake sites. Moreover, binding to uptake sites is non-saturable and cannot be displaced by non-radioactive ligand (Bylund, 1992). The finding that [^{3}H]-CNQX binding was saturable and could be displaced by non-radioactive competitor therefore further suggests that [^{3}H]-CNQX is binding to receptors and not to uptake sites.

CHAPTER 2

AMPA RECEPTOR REGULATION BY AGONIST AND DEPOLARIZING STIMULI

Introduction

One mechanism by which neuronal function can be regulated is by modifications in the elements of synaptic transmission. Modifications of synaptic transmission can take place at the pre- or postsynaptic elements of the synapse. Presynaptically, such regulation can occur through the regulation of neurotransmitter synthesis (Wurtman and Fernstrom, 1976), release (Greengard et al., 1993), or uptake (Kanai et al., 1993). Postsynaptically, modification may occur by regulation of neurotransmitter inactivation (Bird and Aghajanian, 1975) or alterations in neurotransmitter receptor characteristics (Shaw et al., 1994). As neurotransmitter binding to target receptors represents the first crucial step in postsynaptic signal transduction, this chapter will focus on alterations in AMPA receptor binding properties following treatment with stimuli designed to mimic some aspects of synaptic transmission. These stimuli are (1) application of the agonist AMPA to mimic neurotransmitter evoked receptor modification and (2) cellular depolarization using veratridine intended to produce an independent means of assessing current/voltage effects in the absence of direct receptor stimulation.

Receptor regulation has often been observed in response to two key features of synaptic transmission, including agonist binding at receptors and neurotransmitter-induced conductance changes for specific ions. Much ongoing research has employed experiments designed to mimic some aspects of synaptic neurotransmission, using agonist and chemically-induced increases in cellular depolarization. Various receptor populations, including the $GABA_A$, kainate, and

muscarinic ACh (mACh) receptors have been shown to respond to agonist and depolarizing stimuli by a change in the number of functional receptors.

Neurotransmitter Receptor Response to Agonist Stimulation

Ionotropic Receptors

Both $GABA_A$ and kainate receptor populations have been reported to show functional modifications in response to treatment with agonists (Lanius and Shaw, 1993; Mehta and Ticku, 1992; Shaw and Scarth, 1991; Hablitz et al., 1989; Tehrani and Barnes, 1988; Maloteaux et al., 1987). Hablitz et al. (1989) and Tehrani and Barnes (1988) demonstrated that treatment with 100 μM GABA for seven days in cultures of cerebral neurons prepared from chick embryos reduced [^{3}H]-flunitrazepam binding by approximately 70% and led to a significant reduction in the GABA-gated Cl⁻ uptake. This reduction was due to a decrease in receptor number rather than affinity and could be abolished by the concomitant exposure to the GABA receptor antagonist R 5135, suggesting that $GABA_A$ receptor occupancy by agonist was required for such regulation. Similar results were reported by Maloteaux et al. (1987) who found a reversible decrease in the B_{max} value of [^{3}H]-flunitrazepam binding in rat cultured forebrain neurons after 48 hour incubations with 1 mM GABA or 0.1 mM muscimol ($GABA_A$ agonist). Forty-eight hour incubations with GABA also led to 31% and 23% decreases in the B_{max} values of [^{3}H]-muscimol and [^{35}S]-butyl bicyclophosphorothionate (TBST) ($GABA_A$ receptor-associated ion channel antagonist), respectively. Since these decreases in the number of benzodiazepine and $GABA_A$ receptors were measured after homogenization, the GABA or muscimol-induced decreases of specific binding appeared to represent a true receptor loss rather than an internalization or sequestration of the receptors (Maloteaux et al., 1987).

Such a receptor loss could be explained by agonist-induced internalization of the receptor followed by receptor degradation intracellularly.

The above results are supported by another study which reported a significant decrease in GABA$_A$ receptor number in cultured cortical neurons after treatment with 500 μM GABA for five days (Mehta and Ticku, 1992). The level of [^{3}H]-GABA binding declined as a function of the duration of GABA exposure with a maximal decrease of 40% occurring at 5 days of treatment. Twenty percent decreases were observed as early as after 24 hours of treatment. Moreover, a 35% decrease in TBST binding was seen after treatment with 500 μM GABA for five days.

On a faster time scale, Shaw and Scarth (1991) reported a 12% decrease in GABA$_A$ receptor number in adult rat cortical slices following 2 hour incubations with 10 μM muscimol (GABA$_A$ agonist) as measured by [^{3}H]-SR 95531 (GABA$_A$ antagonist) binding. Similar to the inhibitory GABA$_A$ receptors, the excitatory kainate receptor population in adult rat cortical slices showed 26% decreases in receptor number following 2 hour incubations with 10 μM kainate as assessed by [^{3}H]-kainate binding (Lanius and Shaw, 1993).

G-protein Coupled Receptors

The G-protein-coupled mACh receptors have also been reported to decrease in number following treatment with agonist for several hours to days (Shaw et al., 1989; Klein et al., 1979; Siman and Klein, 1979). Activation of this receptor population with 100 μM ACh for 12 hours on neuron-like NG108-15 hybrid cells reduced the number of muscarinic ACh receptors by 88% as assessed by [^{3}H]-quinuclidinyl benzilate (QNB) binding (Klein et al., 1979). This decrease in receptor number remained stable

for up to three days. Withdrawal of ACh resulted in a slow increase in mACh receptor number that could be blocked by concomitant exposure to cycloheximide, a protein synthesis inhibitor. These findings suggested that receptor breakdown/down-regulation was the mechanism underlying the observed receptor regulation (Klein et al., 1979).

On a faster time scale, Siman and Klein (1979) reported a 35% decrease in [^{3}H]-QNB binding in cultured embryonic chicken cerebrum cultures following 90 minute exposures with 1 mM carabachol, a muscarinic receptor agonist. This decrease in binding further decreased to 60% after 9 hours and remained stable for 4 days. The decreases in [^{3}H]-QNB binding observed in response to treatment with carbachol could be partly inhibited by cytochalasin B, a microfilament disrupter. Since cytochalasin B is known to disrupt filaments thereby blocking endocytosis, the decreases in [^{3}H]-QNB binding appeared to partially result from internalization of the receptors (Siman and Klein, 1979). In adult rat cortical slices, 2 hour exposures to 10 μM carbachol led to 26% and 36% decreases in surface and total mACh receptor number as determined by [^{3}H]-N-methyl scopolamine (NMS) and [^{3}H]-QNB binding, respectively (Shaw et al., 1989). Such regulation was highly dependent on temperature with no regulation occurring at temperatures below 30°C.

It was recently reported that agonist-induced receptor regulation of human m3 mACh could only occur in the presence of carboxyl-terminal threonine residues (Yang et al., 1994). Exposure of the human m3 mACh receptor expressed in Chinese hamster ovary cells to 1 mM carabachol for 4 hours resulted in a 20% decrease in [^{3}H]-scopolamine binding. This carbachol-induced decrease in [^{3}H]-scopolamine binding could be abolished by site-directed mutagenesis of the threonine residues (Thr^{550}, 553, 554) present in the receptor cytoplasmic carboxyl terminal. Since these

threonine sites represent possible phosphorylation sites for PKC, it is possible that receptor phosphorylation may play a role in mACh receptor regulation.

<u>Neurotransmitter Receptor Response to Cellular Depolarization</u>

<u>Ionotropic Receptors</u>

Both kainate and $GABA_A$ receptor populations have been reported to show functional modifications in response to treatment with veratridine, an agent which leads to depolarization by blocking sodium channel inactivation (Catterall, 1980). For the kainate receptor population the response to veratridine was similar to that observed in response to agonist stimulation (Lanius and Shaw, 1993). In adult rat cortical slices, treatment with 10 μM veratridine for up to two hours led to 55% decreases in kainate receptor number as assessed by [^{3}H]-kainate binding. In contrast, the inhibitory $GABA_A$ receptor population in rat cortical slices responded to veratridine treatment for two hours by showing a 58% increase in receptor number as measured by [^{3}H]-SR 5531 binding (Shaw and Scarth, 1991). These results are supported by Tabuteau et al. (1993) who reported increases in [^{3}H]-SR 95531 binding in response to 3 hour treatments with a variety of depolarizing agents in rat hippocampal slices. Depolarization induced by treatment with K$^+$, veratridine, v+g, and ouabain led to 72, 113, 127, and 215% increases in [^{3}H]-SR 95531 binding, respectively.

<u>G-protein Coupled Receptors</u>

Liles and Nathanson (1987) reported that incubation with 50 μM veratridine for 24 hours induced a 200% increase in muscarinic receptor number in neuroblastoma cells as determined by [^{3}H]-QNB binding. Increases in mACh number seen as a result of

this treatment could be blocked by tetrodotoxin (TTX) and could be returned to control levels within 20 hours upon withdrawal of veratridine. Chronic membrane depolarization induced by incubation in a medium containing 60 mM potassium chloride led to a TTX-insensitive 50% increase in mACh receptor number after 24 hours. These results are supported by a recent study which demonstrated an increase in BODIPY® FL (muscarinic M_1 receptor-selective antagonist) binding following 7 day treatments with 40 mM potassium chloride in cultured rat visual cortex neurons (Wang et al., 1994). BODIPY® FL binding showed 58% and 40% increases in dendritic processes and cell bodies, respectively.

On a faster time scale, Luqmani et al. (1979) reported a 17% decrease in [^{3}H]-N-methylatropine (NMA) (mACh antagonist) binding in response to 30 minute stimulations with 76 μM veratridine in synaptosomes prepared from rat cerebral cortex through an unknown mechanism. The decrease in [^{3}H]-NMA was not a result of neurotransmitter-receptor interactions as the phenomenon was not achieved by treatment of synaptosomes with the agonist carbachol (0.1-100 μM). Furthermore, K^+ depolarization did not decrease [^{3}H]-NMA binding, suggesting that the decrease in [^{3}H]-NMA binding observed was specifically associated with the opening of Na^+ channels. Similarly, treatment of rat cortical slices with 10 μM veratridine for 2 hours resulted in 26% and 11% decreases in [^{3}H]-NMS and [^{3}H]-QNB binding, respectively (Shaw et al., 1989).

In summary, the above studies have shown that both ionotropic and G-protein coupled receptors can be regulated in response to agonist- and pharmacologically-induced depolarization. The consequences of such regulation, however, can be diverse depending on the specific receptor or stimulus involved.

Materials and Methods

A Brief Overview of Methods

Brain slices were prepared as described previously in the General Methods. The cortical slices were exposed to agonist (AMPA) or veratridine + glutamate (v+g) treatment at 37°C. Following incubation with these substances, the slices were rinsed and incubated with radioligand at 4°C as described previously.

Experiments Designed to Study the Effects of
Agonist and Depolarizing Stimuli on [^{3}H]-CNQX Binding

In order to study regulation of the AMPA receptor population by agonist or depolarizing stimuli, AMPA and v+g were used.

Compound	Concentration (M)
AMPA	10^{-5}
veratridine	10^{-5}
glutamate	10^{-5}

Table 2: Compounds used to study AMPA receptor regulation

AMPA was used to achieve direct activation of the AMPA receptor. In areas of the brain where the GluR2 subunit is not present, activation of AMPA receptors leads to an influx of both Na^+ and Ca^{2+}. However, in brain regions which express GluR2, activation of AMPA receptors leads to an influx of Na^+ currents through AMPA receptor-associated ion channels, only (Hollmann et al., 1991). Since GluR2 is highly

expressed in cortex (Martin et al., 1993; Petralia and Wenthold, 1992), activation of AMPA receptors in cortex only results in an influx of Na^+ through AMPA receptor-associated ion channels. However, membrane depolarization as a result of AMPA receptor-mediated Na^+ influx has been shown to lead to the activation of voltage-gated Ca^{2+} channels with a concomitant influx of Ca^{2+} (Church et al., 1994).

A combination of veratridine and glutamate (v+g) was utilized to produce cellular depolarization. Glutamate increases inward sodium currents via AMPA, kainate, or NMDA receptors, resulting in membrane depolarization and the opening of voltage-gated Na^+ and Ca^{2+} channels; veratridine acts to keep the voltage-gated Na^+ channels open by blocking their inactivation (Catterall, 1980).

Concentrations of 10^{-5} M were chosen for AMPA, veratridine, and glutamate. Dose-response curves had shown these concentrations to be sufficient to obtain maximum effects over the time period examined (see Results). Incubation with both compounds took place for a minimum of 25 min. Time course experiments had shown these incubation times to be most effective to obtain maximum effects (see Results). All regulation experiments were carried out at 37°C, since it has previously been shown that maximum regulatory effects are obtained at physiological temperatures (Van Huizen et al., 1989).

Control Experiments to Distinguish between Competition and Regulation Effects of Veratridine and AMPA

For experiments designed to measure receptor regulation by agonists or veratridine, a key control is to establish the length of rinse necessary to remove all of the agonist or veratridine present. Veratridine has been shown previously to compete for muscarinic

ACh (Van Huizen et al., 1988; Shaw et al., 1989), GABA$_A$ (Shaw and Scarth, 1991),
and AMPA receptor (Lanius and Shaw, 1992) binding by unknown mechanisms. In
addition, AMPA has been shown to be able to compete for [^{3}H]-CNQX binding (see
Chapter 1). Failure to determine this rinse time can lead to confusion between
agonist/veratridine-induced regulation and residual agonist/veratridine competition
with the radioligand. Further, all stages in such determination must be made at low
temperatures (4°C) to prevent regulation during the application of the agonist or
veratridine and to further prevent any possible 'reregulation' during the rinse out
phase (Van Huizen et al., 1989). In order to distinguish between possible competition
and regulation effects, post-AMPA or veratridine rinse times required to return binding
to control levels were measured at 4°C. Rinse times which were adequate to eliminate
any competitive effects of these compounds were subsequently used.

To determine whether the changes in [^{3}H]-CNQX binding observed as a result of
AMPA or v+g treatment were reversible, adult rat cortical slices were incubated with
10^{-5} M AMPA or 10^{-5} M v+g for a minimum of 25 min at 37°C. The slices were then
rinsed twice for 30 min with cold Dul$^+$ after which fresh Dul$^+$ was added for 5 to 45 min
at 37°C. Radioligand binding was then carried out as described previously.
Experiments designed to measure possible internalization of AMPA receptors after
regulation with AMPA or v+g compared [^{3}H]-CNQX binding in slices which were
freeze/thawed to ones which remained intact after regulation with AMPA or v+g. Since
[^{3}H]-CNQX only labels surface receptors in an intact slice preparation (see Chapter 1),
the comparison of [^{3}H]-CNQX binding in intact to freeze/thawed cortical slices should
demonstrate whether AMPA and v+g result in the internalization or sequestration of
the receptors. If the receptors were indeed internalized as a result of AMPA or v+g
treatment, [^{3}H]-CNQX binding in the freeze/thawed slices should not be different from
control. For this experiment, cortical slices were incubated with 10^{-5} M AMPA or 10^{-5}

M v+g for 25 min at 37 °C. Half of the slices were then rinsed twice for 30 min with cold Dul+, frozen to -30°C, and rapidly thawed. The other half was left intact for the radioligand binding step. Radioligand binding was then carried out as described previously.

Neurotoxicity of AMPA and Glutamate

Although it has long been known that the neurotransmitter glutamate and its analogues kainic acid, NMDA, and AMPA can act as neurotoxins (Brorson et al., 1994; Koh et al., 1990; Siman and Card, 1988; Frandsen and Schousboe, 1987), controversy in the literature exists about which conditions are necessary and/or sufficient to produce neuronal damage or cell death. Frandsen and Schousboe (1987) have reported neurotoxic effects of AMPA (ED$_{50}$ 10 μM) in cultured cerebral cortex neurons after exposure times of 1-10 min as assessed by the release of the cytoplasmic enzyme lactate dehydrogenase. Moreover, Brorson et al. (1994) showed that 20 min exposures of cerebellar cultures to AMPA (30 μM) resulted in 25% cell death compared to control cultures as assessed by a fluorescent cytotoxicity assay. In contrast, Koh et al. (1990) suggested that AMPA concentrations as high as 1mM led to only very slow neuronal degeneration in cultured embryonic cerebral cortex neurons. Continuous exposures for up to 24 h were required to elicit substantial cell loss.

Glucose and magnesium have been shown to act as strong neuroprotective agents against excitotoxic compounds (Cox et al., 1989; Lysko et al., 1989; Finkbeiner and Stevens, 1988; Hahn et al., 1988). Cox et al. (1989) reported that cerebellar neurons in primary culture were resistant to a 30 min exposure to glutamate concentrations as high as 5 mM in the presence of 5 x 10^{-5} M glucose and 1 x 10^{-3} M magnesium. Lysko et al. (1989) suggested that cultured cerebellar granule cells were resistant to

40 min exposures of glutamate at concentrations as high as 5 mM in the presence of 10^{-3} M glucose and 5.6×10^{-3} M $MgCl_2$. Moreover, Finkbeiner and Stevens (1988) found 10 μM concentrations of glutamate to be non-toxic in the presence of at least 1.8 $\times 10^{-3}$ M $MgCl_2$ in cultured hippocampal neurons of the CA1 region. Finally, Hahn et al. (1987) reported that 16 h exposures of 0.5×10^{-3} - 5×10^{-3} M glutamate in the presence of 1.8×10^{-3} M Ca^{2+} and 8×10^{-4} M $MgCl_2$ did not result in glutamate-induced cell death of rat retinal ganglion cells. Du^{+}, the medium used in the present experiments, contained 4.7×10^{-2} M $MgCl_2$ as well as 5.6×10^{-3} M glucose. As the concentration of both substances was higher than that used by the studies cited above, the treatments employed in the present experiments should not have resulted in extensive neural death.

2-Deoxy-D-[^{14}C]Glucose Uptake Studies

In order to determine whether treatments with AMPA or glutamate resulted in cell death in the present preparation, viability studies examining the uptake of 2-deoxy-D-[^{14}C]glucose (2-DG) were performed (Sokoloff et al., 1977). 2-DG is an analogue of glucose which differs from glucose in the replacement of the hydroxyl group on the second carbon atom by a hydrogen atom. 2-DG competes with glucose for the glucose carrier that transports glucose into the tissue. Once 2-DG has entered the tissue, its metabolism is identical to that of glucose until a point in the glycolytic pathway is reached where its irregular structure prevents its further metabolism (Sokoloff et al., 1977).

Cortical slices were incubated with AMPA (10^{-5} M) or glutamate (10^{-5} M) for 25 min to 2h at 37°C. After this incubation, slices were rinsed twice for 10 min with Dulbecco's phosphate buffered saline. Five hundred μL of Dulbecco's phosphate buffered saline

containing 5 μCi/mL [^{14}C]deoxyglucose-6-phosphate (57.2 mCi/mmol) were then added to each cortical slice (Keler and Smith, 1989), and the incubation was allowed to proceed for 30 min at 37°C. The cortical slices were then washed twice for 5 min with Dulbecco's phosphate buffered saline and counted in a LS 6000 IC Beckman scintillation counter.

Results

Regulation Experiments

To determine the most effective concentration of AMPA on [^{3}H]-CNQX binding, a concentration-response curve for AMPA over a concentration range of 10^{-9} M to 10^{-4} M was carried out (Figure 5). Concentrations ranging from 10^{-9} M to 10^{-7} M AMPA had little effect on [^{3}H]-CNQX binding. Concentrations ranging from 10^{-6} M to 10^{-4} M AMPA resulted in 16% to 23% decreases in [^{3}H]-CNQX binding. Maximum effects were observed with 10^{-5} M and 10^{-4} M AMPA. A concentration of 10^{-5} M was therefore employed in regulation experiments.

To evaluate the most effective concentration of v+g on [^{3}H]-CNQX binding, a concentration-response curve for v+g over a concentration range of 10^{-9} M to 10^{-4} M (for both compounds) was carried out (Figure 6). Maximum effects were observed with 10^{-5} M and 10^{-4} M v+g (16% and 18% decreases in [^{3}H]-CNQX binding, respectively). A concentration of 10^{-5} M was therefore employed in regulation experiments.

Time course experiments examining specific [^{3}H]-CNQX binding as a function of increasing incubation times with 10^{-5} M AMPA at 37°C were conducted to establish

the incubation time most effective in inducing AMPA receptor regulation by AMPA (Figure 7). [^{3}H]-CNQX binding was decreased by 28% following a 15 min incubation time; this effect remained relatively stable for up to 2 h. A minimum incubation time of 15 min was therefore employed.

Time course experiments examining specific [^{3}H]-CNQX binding following the withdrawal of AMPA were carried out to determine whether the decrease in [^{3}H]-CNQX binding observed as a result of treatment with 10^{-5} M AMPA was reversible (Figure 8). The decrease in [^{3}H]-CNQX binding observed as a result of AMPA treatment was fully reversible upon withdrawal of AMPA. Following washout of AMPA, [^{3}H]-CNQX binding gradually increased, reaching control values by approximately 20 min.

Time course experiments examining specific [^{3}H]-CNQX binding as a function of increasing incubation times with 10^{-5} M v+g at 37°C were carried out to establish the incubation time most effective in inducing AMPA receptor regulation by v+g (Figure 9). [^{3}H]-CNQX binding was decreased by 18% following a 25 min incubation time; this effect remained relatively stable for up to 2 h. A minimum incubation time of 25 min was therefore employed.

Time course experiments examining specific [^{3}H]-CNQX binding following the withdrawal of v+g were conducted to determine whether the decrease in [^{3}H]-CNQX binding observed as a result of treatment with 10^{-5} M v+g was reversible (Figure 10). The decrease in [^{3}H]-CNQX binding observed as a result of v+g treatment was fully reversible upon withdrawal of v+g. Following washout of v+g, [^{3}H]-CNQX binding gradually increased, reaching control values by approximately 20 min.

Experiments designed to distinguish between possible competition and regulation effects of AMPA and v+g showed that by 60 min (2 x 30 min rinses) at 4°C, [^{3}H]-CNQX binding had returned to levels not significantly different from control under either of these conditions (Figure 11). In subsequent regulation experiments, a rinse time of this duration was therefore routinely employed.

Figure 12 shows the effects of 10^{-5} M AMPA or v+g on [^{3}H]-CNQX binding. Both AMPA and v+g significantly decreased binding by an average of 25% and 20%, respectively (p<0.05, Student's t-test). Similar decreases in binding were observed when the cortical slices were freeze/thawed after regulation with AMPA or v+g, suggesting that the mechanism underlying the regulation of these substances was not occurring through internalization of the receptors.

2-Deoxy-D-[^{14}C]Glucose Uptake Studies

Results of 2-deoxy-D-[^{14}C]glucose uptake studies showed that 2-deoxyglucose uptake levels in control slices remained stable for 2 h and decreased by approximately 25% after 6 h. Slices which were frozen to -30°C and then rapidly thawed, a process which kills cells by rupturing their cellular membranes, showed significantly decreased 2-deoxyglucose uptake levels as compared to control (p<0.05, Student's t-test). Exposure of cortical slices to 10 μM AMPA or glutamate for 25 min or 2h did not result in statistically significant different 2-deoxyglucose uptake levels from control slices (p<0.05, Student's t-test) (see Figure 13). One may have expected an increase in 2-deoxyglucose uptake levels after increases in synaptic activity due to stimulation with AMPA or glutamate. However, the slices were exposed to 2-deoxyglucose after they were rinsed for 2 x 10 min following exposure to AMPA or glutamate. It is therefore likely that synaptic activity returned to control levels by this time.
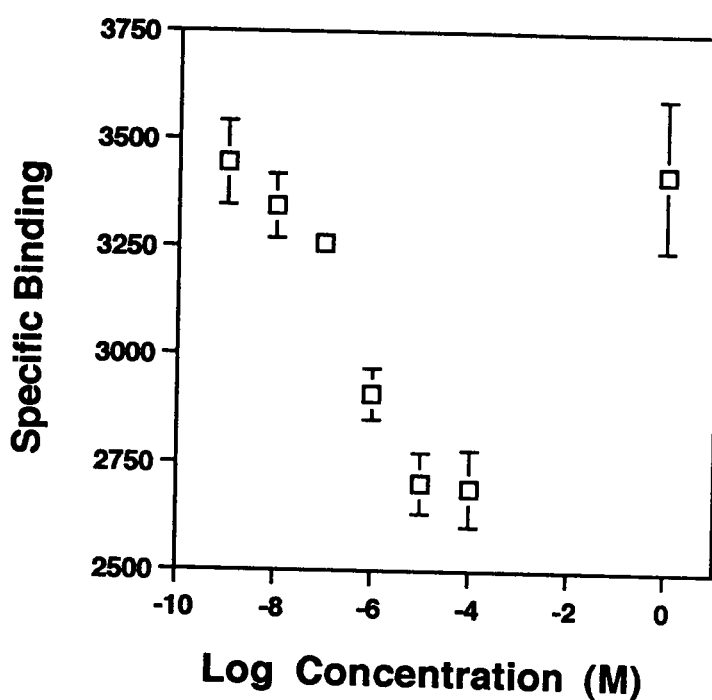
Figure 5: Concentration response of AMPA. Specific [^{3}H]-CNQX binding is plotted as a function of increasing AMPA concentrations. Ligand concentration was 5-10 nM. All incubations with [^{3}H]-CNQX were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M. (n=3). Specific binding is expressed as dpm.
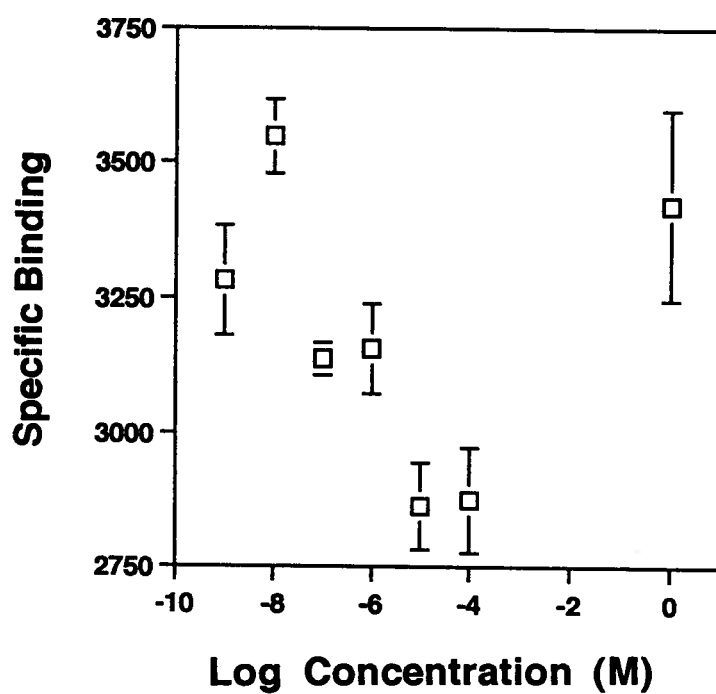
Figure 6: Concentration response of v+g. Specific [^{3}H]-CNQX binding is plotted as a function of increasing v+g concentrations. Assays were carried out as described in Figure 5. Error bars give S.E.M. (n=3). Specific binding is expressed as dpm.
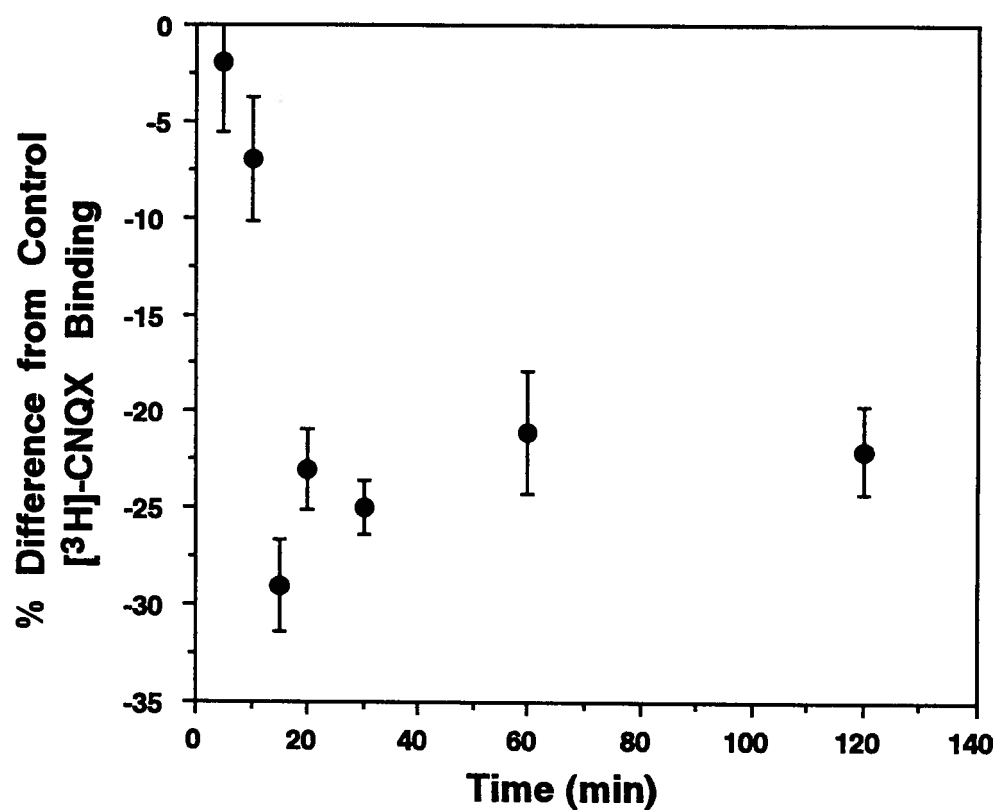
Figure 7: Time course of AMPA-induced regulation of [^{3}H]-CNQX binding. Ligand concentration was 5-10 nM. All incubations with [^{3}H]-CNQX were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M. (n=3).
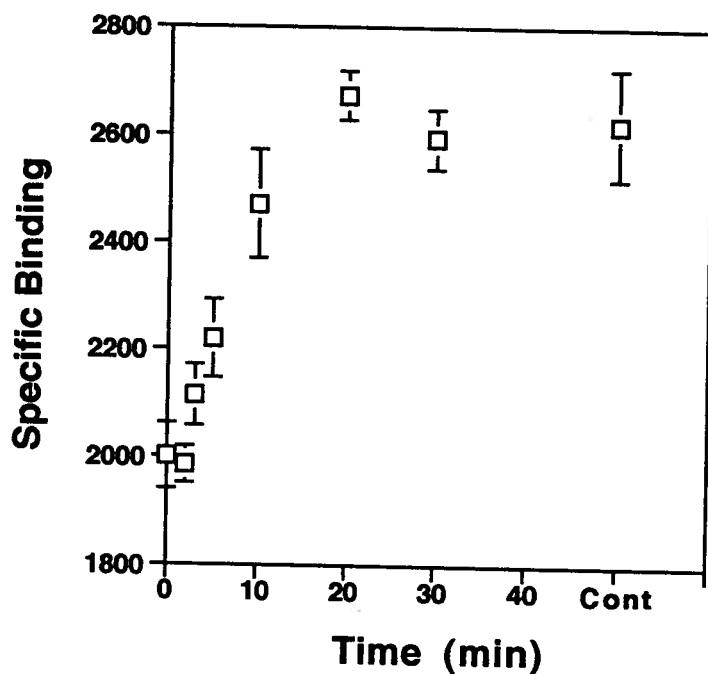
Figure 8: Reversibility of AMPA-induced regulation of [^{3}H]-CNQX binding following withdrawal of AMPA. Cortical slices were incubated with 10^{-5} M AMPA for 25 min at 37°C. The slices were then rinsed twice for 30 min with cold Dul$^+$ after which fresh Dul$^+$ was added for 5 to 45 min at 37°C. All incubations with [^{3}H]-CNQX were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M. (n=3). Specific binding is expressed as dpm.
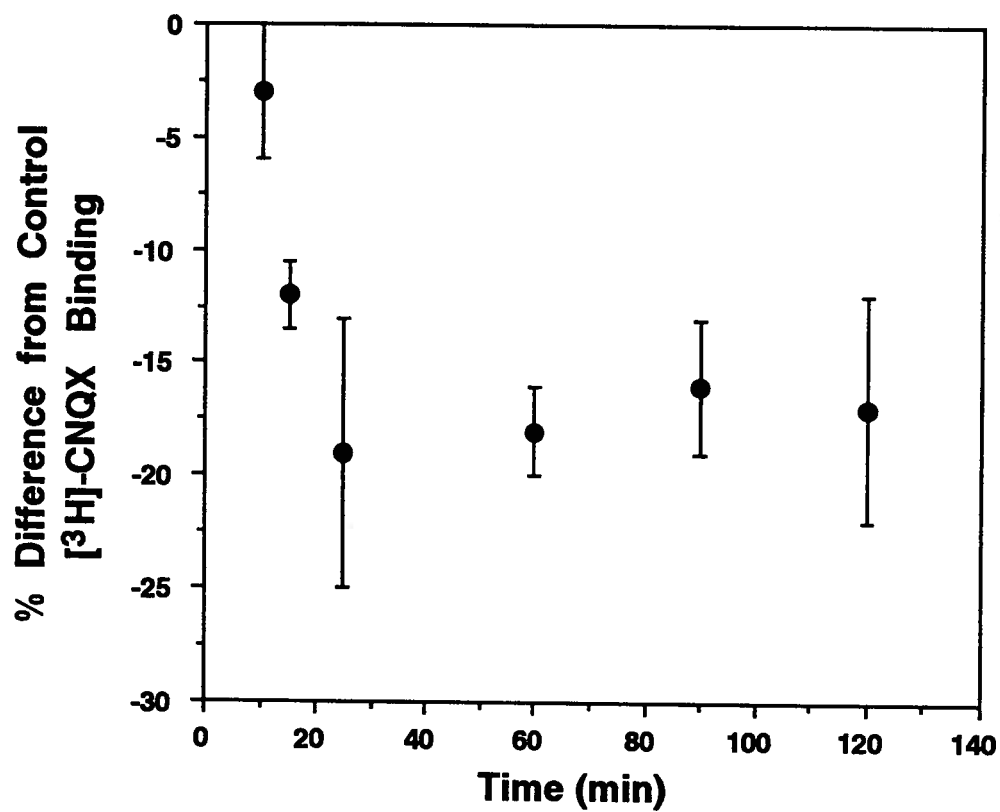
Figure 9: Time course of v+g-induced regulation of [3H]-CNQX binding. Ligand concentration was 5-10 nM. All incubations with [3H]-CNQX were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M. (n=3).
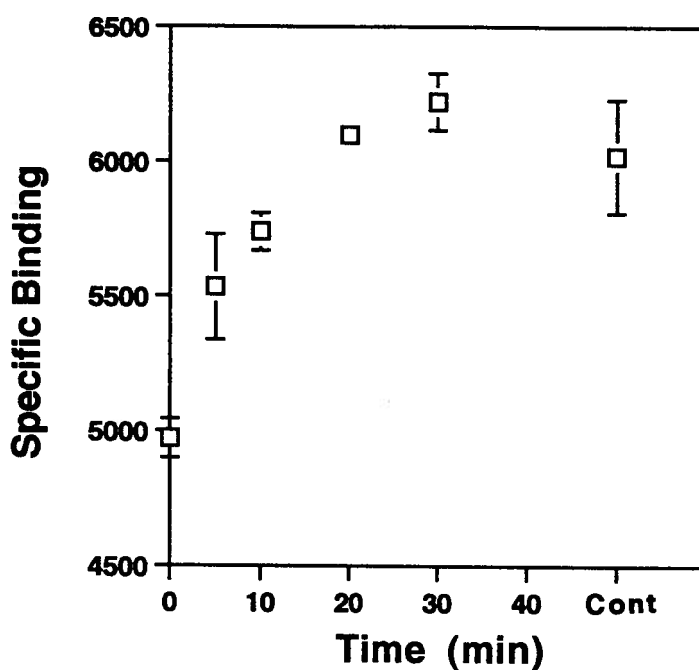
Figure 10: Reversibility of v+g-induced regulation of [^{3}H]-CNQX binding. Cortical slices were incubated with 10^{-5} M v+g for 25 min at 37°C. The slices were then rinsed twice for 30 min with cold Dul$^+$ after which fresh Dul$^+$ was added for 5 to 45 min at 37°C. All incubations with [^{3}H]-CNQX were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M. (n=3). Specific binding is expressed as dpm.
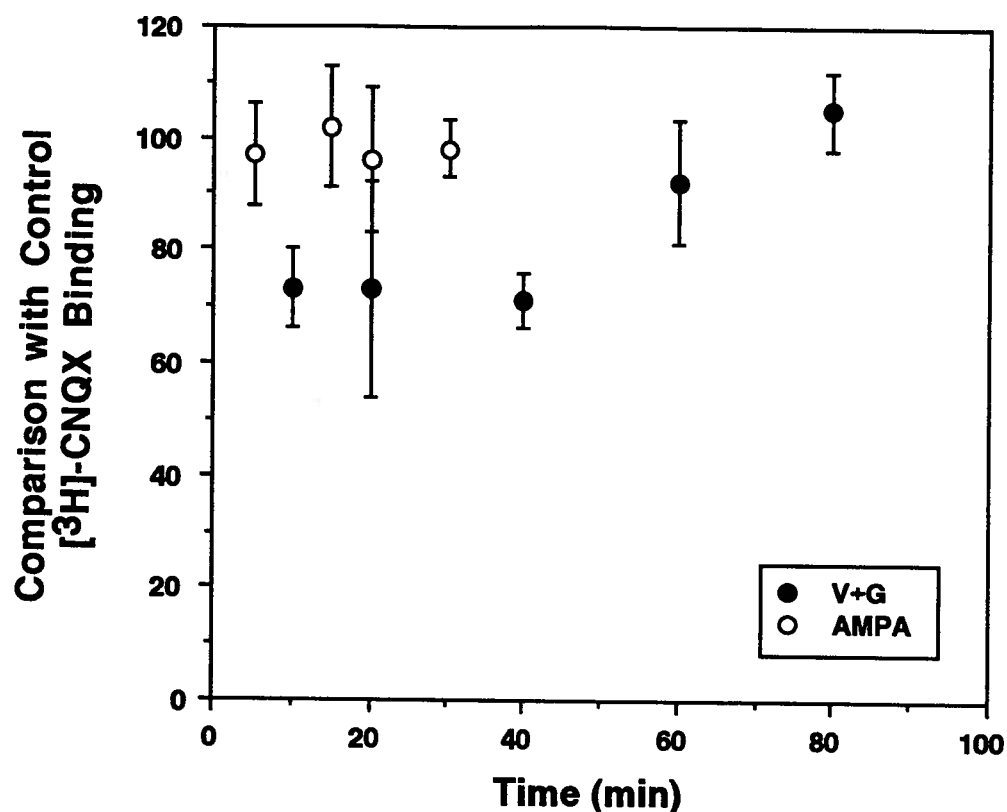
Figure 11: Rinse out times for AMPA or v+g treatments. Control cortical slices were compared to slices treated with 10^{-5}M v+g or AMPA for a minimum of 25 min at 4°C, followed by two 4°C rinses of equal length (10 min to 80 min total time). Following the rinses, the slices were incubated with 5-10 nM [^{3}H]-CNQX for 3 h at 4°C. Slices were rinsed for 2 x 5 min. Error bars give S.E.M. (n=3).
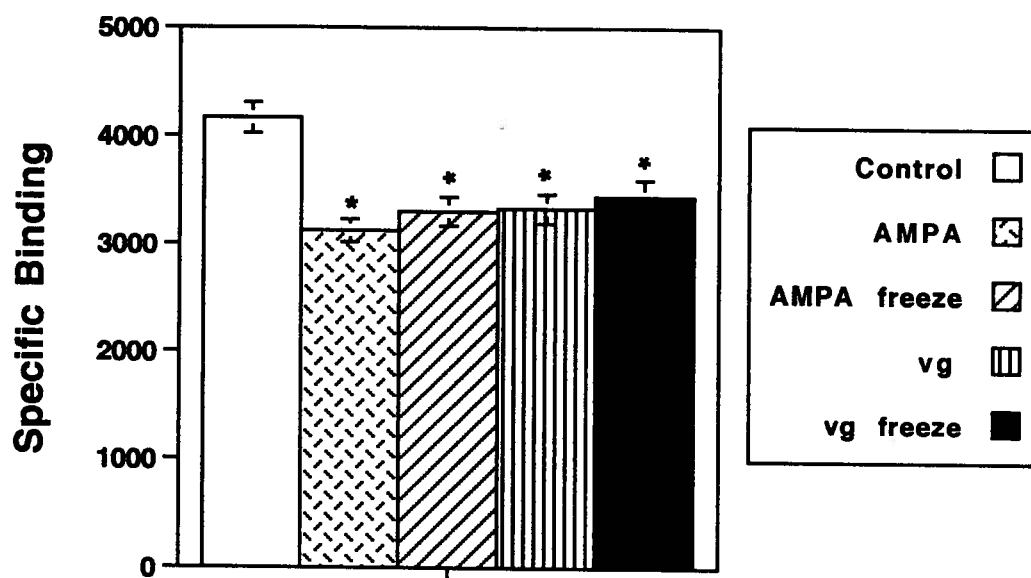
Figure 12: AMPA receptor regulation in response to AMPA or v+g. Rat cortical slices were incubated with AMPA (10^{-5} M) or a combination of v+g (10^{-5} M) for a minimum of 25 min at 37°C. Following this incubation, slices were rinsed for 60 min (2 x 30 min). Slices were then incubated at 4°C with 5-10 nM [^{3}H]-CNQX for 3 h and rinsed for 2 x 5 min. Error bars give S.E.M. (n=3). Asterisks indicate significant differences from control binding to the $p < 0.05$ level (Student's t-test). Specific binding is expressed as dpm.
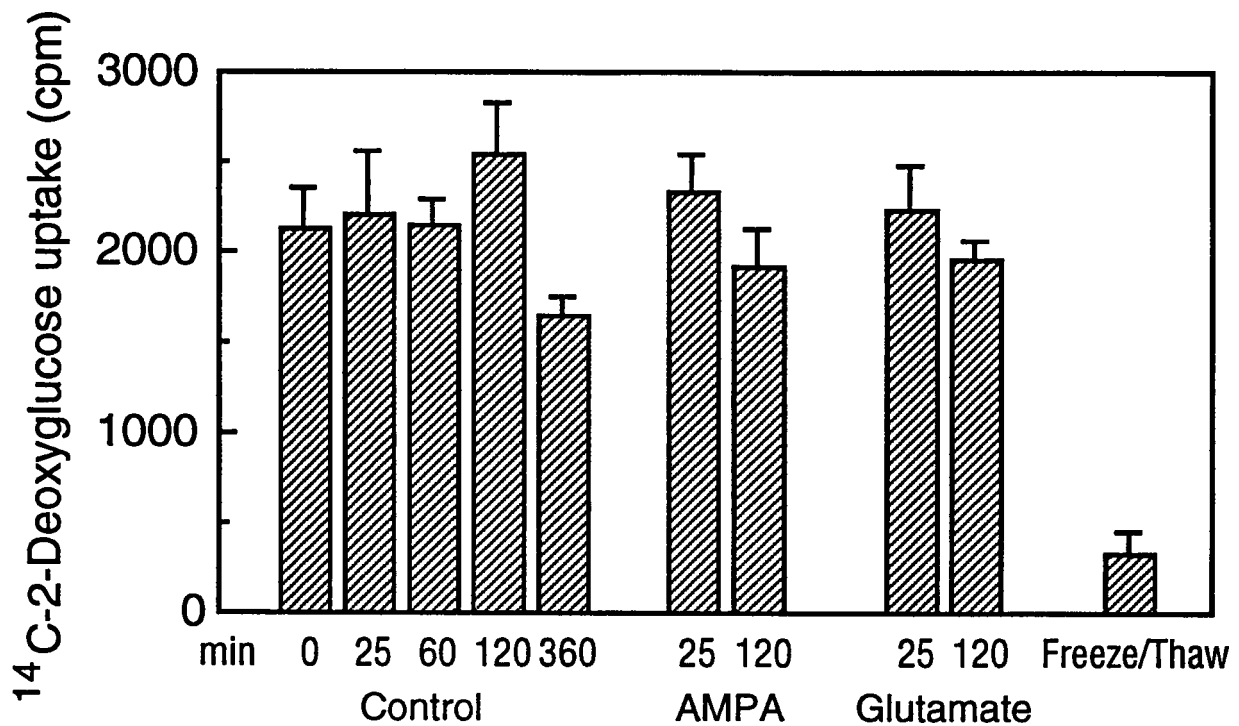
Figure 13: ^{14}C-2-deoxyglucose uptake of cortical slices after treatment with AMPA or glutamate. Cortical slices were incubated with AMPA (10^{-5} M) or glutamate (10^{-5} M) for 25 min or 2h at 37°C. Slices were then rinsed with Dulbecco's phosphate buffered saline for 2 x 30 min. Five hundred μL of Dulbecco's phosphate buffered saline containing 5 μCi/mL [^{14}C]deoxyglucose-6-phosphate were then added to each cortical slice, and the incubation was allowed to proceed for 30 min at 37°C. The slices were then washed twice for 5 min with Dulbecco's phosphate buffered saline. Error bars give S.E.M. (n=5).

Discussion

The preceding regulation experiments have demonstrated reversible decreases in [^{3}H]-CNQX binding in response to 10^{-5} M AMPA treatment. These effects were seen after 15 minutes of AMPA treatment and remained stable for up to two hours. The decreases in binding observed in response to a short duration (minutes to hours) of agonist stimulation were similar in direction to those reported previously for other receptor populations for their own agonists in this and other preparations. Agonist treatment of the ionotropic GABA$_A$ and kainate receptors for 2 hours led to 12% and 26% decreases in GABA$_A$ (Shaw and Scarth, 1991) and kainate (Lanius and Shaw, 1993) receptor number, respectively. Similarly, agonist treatment of the G-protein coupled mACh receptors for 1.5 and 4 hours has been reported to result in 20% (Yang et al., 1994) and 36% (Siman and Klein, 1979) decreases in mACh receptor number, respectively. Although the mechanism underlying such regulation has remained largely unknown, evidence has suggested that both receptor phosphorylation (Yang et al., 1994) as well as receptor internalization (Siman and Klein, 1979) may be partially responsible (see Introduction).

Agonist treatment for many hours to days resulted in decreases in receptor binding which were, on average, greater in magnitude than the ones observed after a shorter duration of agonist treatment. Agonist treatment of GABA$_A$ receptors for 5 and 7 days resulted in 40% (Mehta and Ticku, 1992) and 70% (Hablitz et al., 1989; Tehrani and Barnes, 1988) decreases in GABA$_A$ receptor number, respectively. Agonist treatment of mACh receptors for 12 hours resulted in 88% decreases in mACh receptor number (Klein et al., 1979).

Treatment with v+g (10^{-5} M) led to reversible 17% decreases in [^{3}H]-CNQX binding in a similar manner to the effects of AMPA. These effects were seen after 25 minutes and remained stable for up to two hours. The decreases in binding observed in response to short durations (minutes to hours) of veratridine treatment were akin to those reported for kainate- (Lanius and Shaw, 1993) and muscarinic ACh receptors (Shaw et al., 1989; Luqmani et al., 1979) in the same and other preparations. Exposures of rat cortical slices to 10 μM veratridine led to a 55% decrease in kainate receptor number (Lanius and Shaw, 1993). Similarly, 30 minute and 2 hour exposures to veratridine led to 17% (Shaw et al., 1989) and 26% (Luqmani et al., 1979) decreases in mACh receptor binding, respectively. In contrast, the inhibitory GABA$_A$ receptor population in rat cortical slices showed a 58% <u>increase</u> in receptor number in response to 10 μM veratridine treatment for 2 hours (Shaw and Scarth, 1991). Similarly, treatment of rat hippocampal slices with 10 μM veratridine for 3 hours resulted in 127% increases in GABA$_A$ receptor number as assessed by [^{3}H]-SR 95531 binding (Tabuteau et al., 1993).

It is conceivable that the direction of regulation to increases in cellular depolarization may be linked to the type of activity induced by agonist stimulation. GABA$_A$ agonists increase chloride currents to hyperpolarize neurons, an opposite effect to that induced by v+g. For AMPA and kainate receptors, both agonists and veratridine and glutamate act to increase sodium currents, and both treatments led to a decrease in receptor binding.

We have previously reported that quisqualate- and veratridine-induced changes in [^{3}H]-CNQX binding are likely due to changes in high-affinity AMPA receptor number (Lanius and Shaw, 1992; Shaw and Lanius, 1992). However, this interpretation of results has to be reevaluated. [^{3}H]-CNQX appears to bind to both the high- and low-

affinity component of the AMPA receptor (Honore et al., 1989). It therefore remains unknown whether AMPA receptor regulation induced by AMPA and veratridine is due to the regulation of the high- and/or low-affinity component of [^{3}H]-CNQX binding. Honore et al. (1989) reported that at a concentration of 2 nM, the high-affinity site corresponded to 28% of [^{3}H]-CNQX binding and the low affinity site corresponded to 72% of the binding. Moreover, it remains unknown whether a change in receptor number or affinity is responsible for the observed changes in [^{3}H]-CNQX binding. However, several possibilities seem plausible. (1) It is possible that the decreases in [^{3}H]-CNQX binding observed after treatment with AMPA and veratridine reflect a shift from the high-affinity state to the low-affinity state of the AMPA receptor. (2) An alternative possibility is that AMPA and veratridine lead to a change in receptor number of the high- and/or low-affinity state of the AMPA receptor. (3) A third possibility is that AMPA and veratridine lead to a change in receptor affinity of the high- and/or low affinity state of the AMPA receptor. (4) Lastly, it is conceivable that AMPA and veratridine result in changes in receptor number and affinity of the high- and/or low-affinity state of the AMPA receptor. Since agonist stimulation and increases in cellular depolarization have often been reported to lead to changes in receptor number (see Chapter 2 Introduction), however, it is likely that the changes in [^{3}H]-CNQX binding seen in response to treatment with AMPA and veratridine are due to changes in the number of high- and/or low-affinity AMPA receptors.

Is it possible that the observed AMPA receptor regulation is an artifact of potential competitive effects of AMPA or v+g for [^{3}H]-CNQX binding or, alternatively, could it be an artifact of cell death due to possible neurotoxic actions of these agents? Competitive effects of AMPA or v+g can be excluded, since experiments designed to distinguish between possible competition and regulation effects showed that by 60 minutes at 4°C, [^{3}H]-CNQX binding had returned to levels not significantly different

from control. Moreover, it is unlikely that the decreases in [^{3}H]-CNQX binding in response to AMPA or v+g treatment were the result of cell death. First, viability studies employing 2-deoxy-D-[^{14}C]glucose uptake showed that exposure of cortical slices to 10 μM AMPA or glutamate for up to two hours did not result in significantly different 2-deoxyglucose uptake levels from control slices. Second, the regulatory effects of AMPA and v+g were completely reversible within approximately 20 minutes and therefore are unlikely to be an artifact of cell death.

Although the mechanism underlying the decreases in AMPA receptor number as a result of agonist and depolarizing treatment is not clear at present, several possibilities can be considered: (1) receptor sequestration/ internalization, (2) receptor down-regulation, (3) repression of receptor gene expression with concomitant receptor down-regulation, and/or (4) a change in the phosphorylation state of the receptor which has been previously shown to alter receptor function, including agonist/antagonist binding, for a variety of ionotropic receptors (Kitamura et al., 1993; Krieger et al., 1993; Shaw et al., 1992; Vaello et al., 1992). Receptor sequestration/internalization appears unlikely since there was no difference in [^{3}H]-CNQX binding between intact and freeze/thawed slices. Receptor-down regulation also does not seem to occur, since the decrease in [^{3}H]-CNQX binding was fully reversible within approximately 20 minutes, and *de novo* receptor synthesis is unlikely to occur in such a short period (Collins et al., 1991; Campbell et al., 1991). Furthermore, repression of receptor gene expression with concomitant receptor down-regulation is improbable to account for the decrease in [^{3}H]-CNQX binding, because the decrease in binding was fully reversible within 20 minutes. The possibility that changes in the phosphorylation state of the AMPA receptor are responsible for the decrease in [^{3}H]-CNQX binding observed seems more likely. Phosphorylating agents have been shown to alter agonist/antagonist binding for a variety of receptor

populations, including the $GABA_A$ (Shaw et al., 1992), glycine (Ruiz-Gomez et al., 1991), and NMDA (Kitamura et al., 1993; Krieger et al., 1993) receptors. The role of phosphorylation reactions in AMPA receptor regulation as assessed by $[^3H]$-CNQX binding will therefore be examined in the chapters to follow.

An additional issue which needs to be addressed is whether the regulation induced by AMPA or v+g occurs on neurons and/or glial cells. Studies examining the immunocytochemical localization and expression of AMPA-selective glutamate receptors in cortex have led to contradictory results (Patneau et al., 1994; Martin et al., 1993; Petralia and Wenthold, 1992). Patneau et al. have reported rapidly desensitizing responses to AMPA in rat cortical oligodendrocytes. Moreover, mRNA for GluR 2-4 appeared to be expressed in rat cortical oligodendrocytes (Patneau et al., 1994). In contrast, Petralia and Wenthold have suggested only limited evidence for GluR 1-4 immunoreactivity in cortical glial cells, although cortical astrocytes have recently been shown to exhibit GluR 4 immunoreactivity (Martin et al., 1993). However, since native AMPA receptors in rat brain usually exist as hetero-oligomers composed of two or more of the four GluR subunits (Wenthold et al., 1992), it remains questionable whether GluR 4 subunits alone are able to form functional AMPA receptors. Moreover, studies using cultured rat cortical astrocytes have found $[^3H]$-CNQX binding not to be present on these cells (Lanius et al., unpublished observations). It is, therefore, unlikely that the regulation observed in response to AMPA or v+g treatment is a result of astrocytic AMPA receptor regulation in the present preparation. Nevertheless, future experiments could further resolve this issue by studying AMPA receptor regulation to AMPA or v+g in pure neuronal cell cultures. In summary, data have shown that the AMPA receptor population in adult rat cortex can be regulated by treatment with agonists or v+g, agents that mimic aspects of synaptic neurotransmission *in vivo*. The regulation of these and other receptors (Yang

et al., 1994; Wang et al., 1994; Lanius and Shaw, 1993; Tabuteau et al., 1993 Mehta and Ticku, 1992; Shaw and Scarth, 1991; Hablitz et al., 1989; Shaw et al., 1989; Tehrani and Barnes, 1988; Maloteaux et al., 1987; Liles and Nathanson, 1987; Klein et al., 1979; Luqmani et al., 1979) in neural tissue lend support to the view that receptor regulation may play a key role in the control of neural function. The regulation of receptor binding following agonist or depolarizing stimuli may serve to alter the response to additional neurotransmitter release. This may not only constitute a possible homeostatic mechanism in the control of interneural communication, but may also provide an essential means for controlling future input activity.

CHAPTER 3

AMPA RECEPTOR REGULATION BY PHOSPHORYLATION

Introduction

The previous chapter has shown that stimulation of rat cortical slices by AMPA or v+g decreased $[^3H]$-CNQX binding. However, the mechanism by which such regulation occurs remains unknown. One possibility is that depolarization in response to AMPA or v+g leads to protein phosphorylation and perhaps specific receptor phosphorylation. In support of this view, it has been shown that depolarization induced by veratridine or high external K^+ stimulated the incorporation of ^{32}P into two specific proteins in a rat synaptosomal preparation (Sieghart et al., 1979; Forn and Greengard, 1978). More recent studies have documented the role of phosphorylation reactions in the regulation of many ionotropic receptor properties (for review see Swope et al., 1992; Huganir and Greengard, 1991). Phosphorylating agents have also been shown to alter agonist/antagonist binding for a variety of ionotropic receptor populations, including the $GABA_A$ (Shaw et al., 1992), glycine (Vaello et al., 1992), and NMDA (Kitamura et al., 1993; Krieger et al., 1993) receptors.

Experiments designed to examine the effects of multifunctional protein kinases on $[^3H]$-CNQX binding will be the focus of this chapter. These experiments will test the hypothesis that phosphorylation reactions are involved in the regulation of the agonist binding site of the AMPA receptor population.

As stated in the General Introduction, the functional modification of AMPA receptors by CaMKII, PKC, as well as PKA has been the focus of several studies (McGlade-McCulloh et al., 1993; Keller et al., 1992; Greengard et al., 1991). CaMKII (Tan et al., 1994; McGlade McCulloh et al., 1993), PKC (Tan et al., 1994; McGlade McCulloh et al., 1993) as well as PKA (Keller et al., 1992; Shaw et al., 1992; Greengard et al., 1991) have been reported to be involved in the regulation of the AMPA receptor population in various neural preparations.

Native AMPA receptors have been shown to be phosphorylated by CaMKII in a variety of preparations (McGlade-McCulloh et al., 1993). Activation of endogenous stores of CaMKII led to the phosphorylation of GluR1 in hippocampal postsynaptic densities, synaptosomes and cultured hippocampal pyramidal neurons. PKC only resulted in a slight enhancement of phosphorylation of GluR1, whereas treatment with the catalytic subunit of PKA did not result in the phosphorylation of the GluR1 subunit in any of these preparations. Moreover, activation of endogenous CaMKII led a three- to fourfold enhancement of AMPA receptor-mediated currents in cultured hippocampal neurons. In a further study, Tan et al. (1994) reported that activation of NMDA receptors by treatment of hippocampal neurons with glutamate and glycine increased ^{32}P labeling of immunoprecipitated AMPA receptors by 145% of control values. This increased phosphorylation of the AMPA receptor population was primarily ^{32}P-serine with little ^{32}P-threonine and no detectable ^{32}P-tyrosine, and could be blocked by a NMDA receptor antagonist (AP-5) or by 1-[N,O-bis(1,5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), a membrane-permeable inhibitor of CaMKII. These results suggested that Ca^{2+} influx through the NMDA receptor

population could result in the activation of CaMKII and concomitant phosphorylation and regulation of the AMPA receptor.

In contrast to the above, Keller et al. (1992) reported that bath application of a membrane permeable analogue of cAMP, an activator of PKA, potentiated the currents and also the flux of calcium through AMPA receptor channels comprised of GluR1 and GluR3 subunits expressed in *Xenopus* oocytes. The average current increases were significantly smaller when oocytes were loaded with the PKA inhibitor R_p-adenosine 3',5'-cyclic monophosphothioate (R_p-cAMPS). In addition to the preceding results, PKA had been demonstrated to increase the opening frequency and the mean open time of the AMPA receptor channels in hippocampal pyramidal neurons, an effect which could be completely blocked by a specific PKA inhibiting peptide (Greengard et al., 1991). Other results suggested that exposure of adult cortical rat slices to the catalytic subunit of PKA led to a significant decrease in [^{3}H]-CNQX binding. This effect could be blocked by a specific peptide inhibitor of PKA (Shaw et al., 1992).

Materials and Methods

Brain slices were prepared as described in the General Methods.

Unlike the usual method of slice preparation where every effort was made to keep the slices alive, the slices in the present experiments were frozen to -30°C and then rapidly thawed. Freeze/thaw treatment promotes ice-crystal formation in slices thereby disrupting cellular membranes and permeabilizing cells, thereby allowing phosphorylating enzymes to reach intracellular

phosphorylation sites. Such treatment had no effect on [^{3}H]-CNQX binding (see Chapter 1 and Lanius and Shaw, 1992). Once the slices were thawed, 500 μL Dulbecco's medium containing the following enzymes or enzyme activators was added to each slice:

i) CaMKII activating cocktail (0.5 mM CaCl$_2$, 2 μM calmodulin, 5 μM PKA inhibiting peptide (IP), 2 μM PKC-IP as described by Tan et al., 1994; McGlade-McCulloh et al., 1993)

ii) catalytic subunit of PKA (Sigma, Type III, 20 ng/mL, Stelzer et al., 1988)

iii) 12-O-tetradecanoylphorbol 13-acetate (TPA) (potent PKC activator, 200 nM; Pelech et al., 1991; Cochet et al., 1986; Pelech et al., 1986; Tapley and Murray, 1985; Kraft et al., 1982)

Dose response curves for the various enzymes or enzyme activators were not carried out, since optimal concentrations have been previously determined (see references above). Incubation with the CaMKII activating cocktail or the catalytic subunit of PKA was allowed to proceed for a minimum of 25 min at 37°C. These incubation times were shown by time course experiments to achieve maximal effects (Figures 13 & 14). Incubation with TPA was allowed to continue for 15 min (Tan et al., 1994; Cochet et al., 1986; Pelech et al., 1986) at 37°C. The slices were then rinsed twice for 30 min at 4°C, and radioligand binding was carried out as described as in the General Methods.

To determine whether the changes in [^{3}H]-CNQX binding observed as a result of CaMKII or PKA treatment were reversible, adult rat cortical slices were incubated with CaMKII activating cocktail or the catalytic subunit of PKA for 25 min at 37°C. The slices were then rinsed twice for 30 min with cold Dul+ after

which fresh DuI^+ was added for 5 to 45 min at 37°C. Radioligand binding was then carried out as described in the General Methods.

An additional set of experiments examined whether the effects of CaMKII or PKA on AMPA receptor regulation could be inhibited using selective inhibitors (see Table 3 below). Since TPA had no effect on $[^3H]$-CNQX binding, PKC inhibitors were not used. Dose-response curves for the various kinase inhibitors were not carried out, since optimal concentrations have been previously determined for all these inhibitors in a variety of different preparations (see Table 3 for individual references).

Inhibitor	Specificity	Concentration	References
PKA-IP	PKA	5 μM	1) Tan et al., 1994 2) McGlade-McCulloh et al., 1993 3) Roth et al., 1991 4) Smith et al., 1990
KN-62	CaMKII	50 μM	1) Tansey et al., 1992 2) Ishii et al., 1991 3) Tokumitsu et al., 1990

Table 3: Protein kinase inhibitors

Slices were co-incubated with either the enzyme or enzyme activator as well as the specific inhibitor for that particular enzyme for a minimum of 25 min at 37°C.

After this incubation, the slices were rinsed twice for 30 min at 4°C, after which receptor binding assays were carried out as described in the General Methods.

Results

Time course experiments examining [^{3}H]-CNQX binding as a function of increasing incubation times with a CaMKII activating cocktail at 37°C were carried out to establish the incubation time most effective in inducing AMPA receptor regulation by CaMKII (Figure 14). [^{3}H]-CNQX binding was decreased by 35% following a 20 min incubation time; this effect remained stable for up to 2 h. A minimum incubation time of 20 min was, therefore, employed in subsequent experiments.

Time course experiments examining [^{3}H]-CNQX binding following the withdrawal of a CaMKII activating cocktail were carried out to determine whether the decrease in [^{3}H]-CNQX binding observed as a result of treatment with CaMKII activating cocktail was reversible (Figure 15). The decrease in [^{3}H]-CNQX binding observed as a result of treatment with a CaMKII activating cocktail was reversible upon withdrawal of the CaMKII activating cocktail. Following washout of the CaMKII activating cocktail, AMPA receptor number gradually increased, reaching control values by approximately 30 min.

Time course experiments examining specific [^{3}H]-CNQX binding as a function of increasing incubation times with the catalytic subunit of PKA at 37°C were carried out to establish the incubation time most effective in inducing AMPA receptor regulation by PKA (Figure 16). [^{3}H]-CNQX binding was decreased by 30% following a 25 min incubation time; this effect remained stable for up to 2 h.

A minimum incubation time of 25 min was therefore employed in subsequent experiments.

Time course experiments examining specific [^{3}H]-CNQX binding following the withdrawal of PKA were carried out to determine whether the decrease in [^{3}H]-CNQX binding observed as a result of treatment with the catalytic subunit of PKA was reversible (Figure 17). The decrease in [^{3}H]-CNQX binding observed as a result of treatment with PKA was reversible upon withdrawal of PKA. Following washout of PKA, AMPA receptor number gradually increased, reaching control values by approximately 15 min.

The effects of CaMKII, PKA or PKC treatment on the AMPA receptor population are shown in Figure 18. Activation of endogenous CaMKII led to a significant 33% decrease in [^{3}H]-CNQX binding ($p < 0.05$, Student's t-test) which could be blocked by KN-62 (n=4). Similarly, PKA led to a significant 30% decrease in [^{3}H]-CNQX binding ($p < 0.05$, Student's t-test) which could be blocked by a specific PKA inhibiting peptide (PKAIP) (n=4). Treatment of cortical slices with TPA had no effect on [^{3}H]-CNQX binding (n=3). KN-62 and PKAIP had no significant independent effect on [^{3}H]-CNQX binding (data not shown).
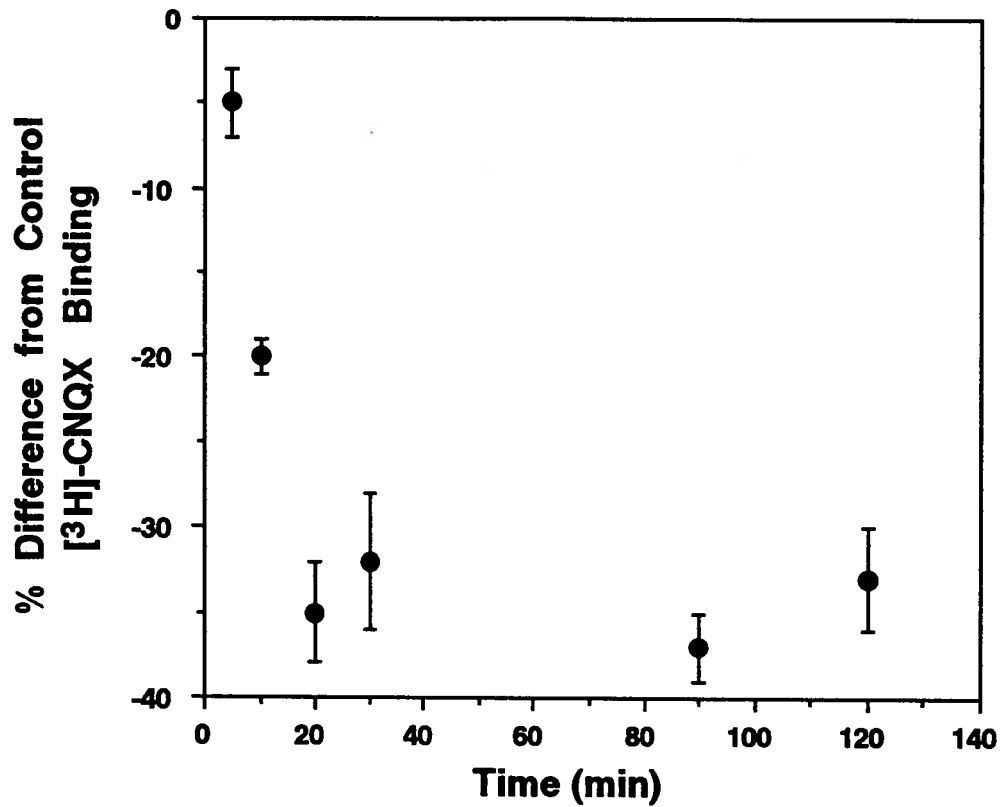
Figure 14: Time course experiments showing [^{3}H]-CNQX binding as a function

of increasing incubation times with a CaMKII activating cocktail. Ligand

concentration was 5-10 nM. All incubations with [^{3}H]-CNQX were for 3 h at 4°C.

Rinse times were 2x5 min. Error bars give S.E.M. (n=3).
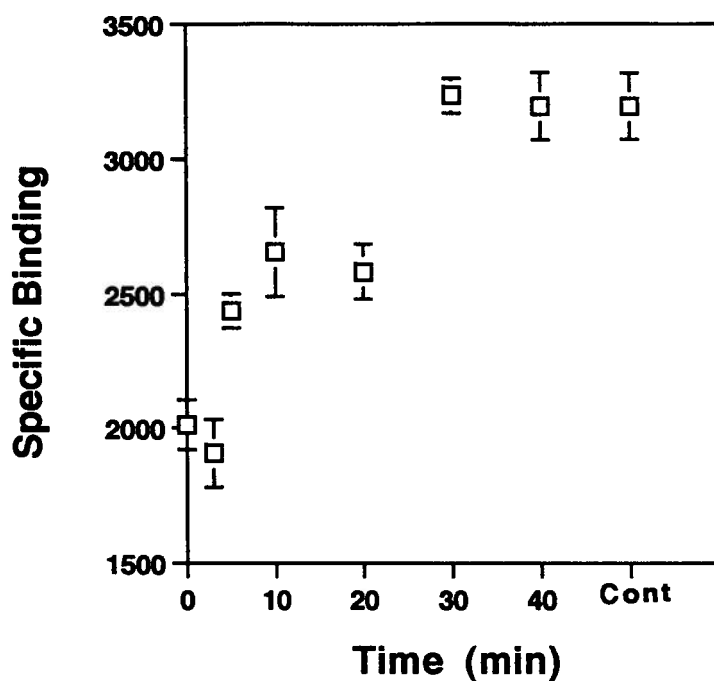
Figure 15: Reversibility of CaMKII-induced regulation of [³H]-CNQX binding.

Cortical slices were incubated with CaMKII activating cocktail for 25 min at

37°C. The slices were then rinsed twice for 30 min with cold DuI⁺ after which

fresh DuI⁺ was added for 5 to 45 min at 37°C. All incubations with [³H]-CNQX

were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M.. Specific

binding is expressed as dpm.
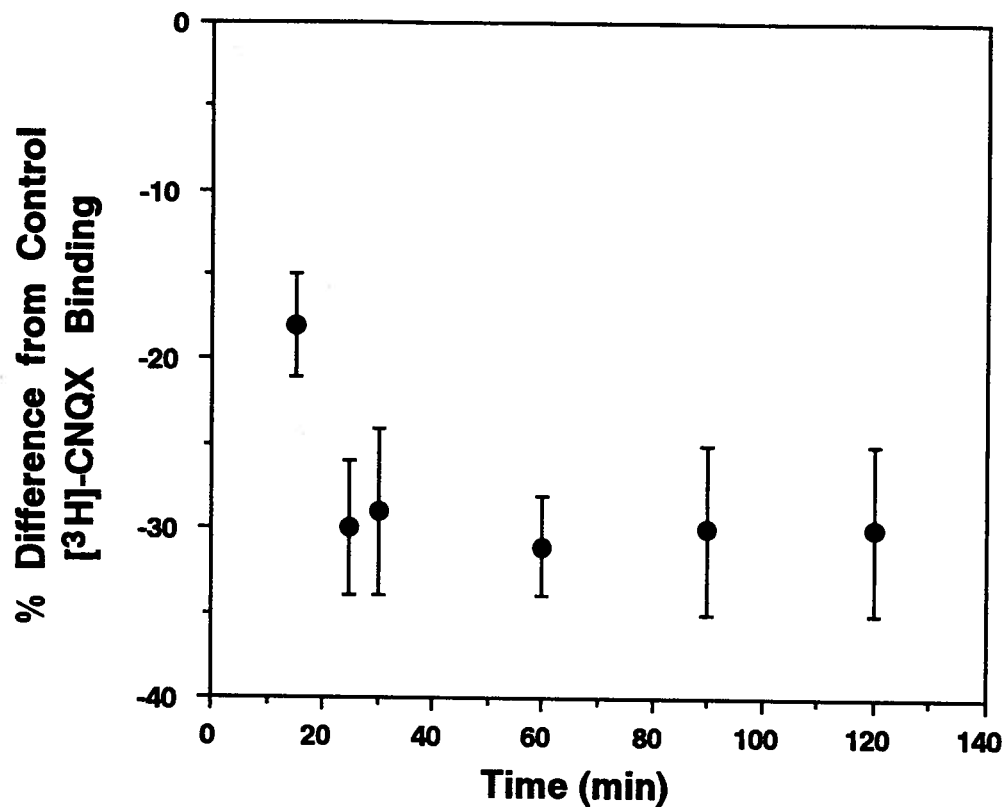
Figure 16: Time course experiments showing [^{3}H]-CNQX binding as a function

of increasing incubation times with the catalytic subunit of PKA. Ligand

concentration was 5-10 nM. All incubations with [^{3}H]-CNQX were for 3 h at 4°C.

Rinse times were 2x5 min. Error bars give S.E.M. (n=3).
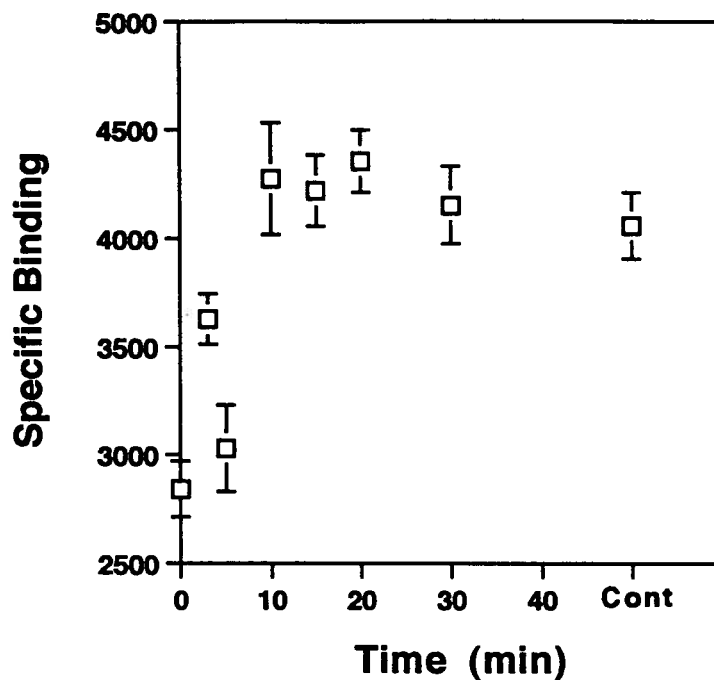
Figure 17: Reversibility of PKA-induced regulation of [³H]-CNQX binding. Cortical slices were incubated with PKA for 25 min at 37°C. The slices were then rinsed twice for 30 min with cold Dul⁺ after which fresh Dul⁺ was added for 5 to 45 min at 37°C. All incubations with [³H]-CNQX were for 3 h at 4°C. Rinse times were 2x5 min. Error bars give S.E.M.. Specific binding is expressed as dpm.

A

B
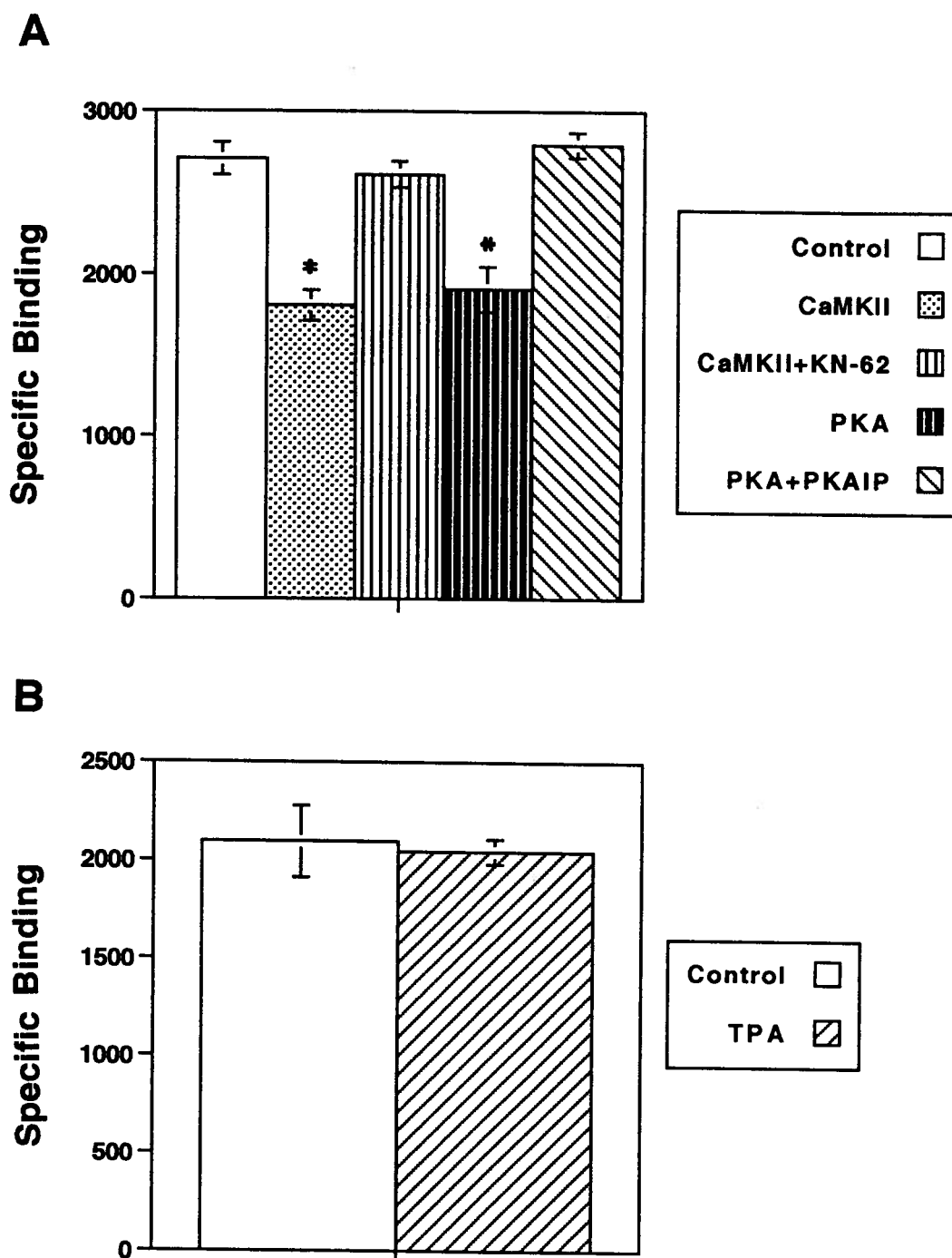
Figure 18: Enzymatic control of the regulation of AMPA receptors labeled with [^{3}H]-CNQX. AMPA receptor binding values were determined after CaMKII, PKA, or TPA treatment alone and in conjunction with selective inhibitors (see Table 3). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the $p<0.05$ level (Student's t-test). Specific binding is expressed as dpm.

Discussion

The preceding regulation experiments have demonstrated reversible decreases in [^{3}H]-CNQX binding in response to a CaMKII activating cocktail and treatment with the catalytic subunit of PKA. These effects were seen within approximately 25 minutes of treatment and remained stable for up to two hours. The actions of these enzymes were selective and could be blocked by the CaMKII inhibitor KN-62 and a specific PKA inhibiting peptide, respectively. Treatment of cortical slices with TPA, an activator of PKC, had no effect on [^{3}H]-CNQX binding.

We have previously reported that PKA-induced changes in [^{3}H]-CNQX binding are likely due to changes in high-affinity AMPA receptor number (Shaw et al., 1992). However, the interpretation of these results has to be reevaluated. It remains unknown whether AMPA receptor regulation induced by CaMKII and PKA is due to the regulation of the high- or low-affinity component of [^{3}H]-CNQX binding. Moreover, it remains unknown whether a change in receptor number and/or affinity is responsible for the observed changes in [^{3}H]-CNQX binding. However, as discussed in Chapter 2, several possibilities seem plausible. (1) It is possible that the decreases in [^{3}H]-CNQX binding observed after treatment with CaMKII and PKA reflect a shift from the high-affinity state to the low-affinity state of the AMPA receptor. (2) An alternative possibility is that CaMKII and PKA lead to a change in receptor number of the high- and/or low-affinity state of the AMPA receptor. (3) A third possibility is that CaMKII and PKA lead to a change in receptor affinity of the high- and/or low affinity state of the AMPA receptor. (4) Lastly, it is conceivable that CaMKII and PKA result in changes in receptor number and affinity of the high- and/or low-affinity state of the AMPA receptor. Experiments using newly developed competitive antagonists which

preferentially bind to the high- or low-affinity component of the AMPA receptor (Ebert et al., 1994) could be used to distinguish whether CaMKII, PKA, AMPA, or veratridine are regulating the high and/or low-affinity component of the AMPA receptor.

Changes in ligand binding as a result of treatment with phosphorylating agents have been reported previously for a variety of receptor populations, including the glycine (Vaello et al. 1992), $GABA_A$ (Shaw et al., 1992) and NMDA (Kitamura et al., 1993; Krieger et al., 1993) receptors. Vaello et al. (1992) examined the effects of PKC-mediated phosphorylation of glycine receptors purified from rat spinal cord on the subsequent interaction of glycine with its receptor. Under control conditions glycine displaced [^{3}H]-strychnine binding with an IC_{50} of 58 μM, whereas for the phosphorylated glycine receptors the IC_{50} was 200 μM. These results indicated a three-fold decrease in the ability of glycine to interact with phosphorylated glycine receptors.

A decrease in [^{3}H]-SR 95531 ($GABA_A$ antagonist) binding was reported following treatment with the catalytic subunit of PKA in rat cortical slices. These effects were attributable to changes in receptor number as indicated by saturation binding analyses and could be blocked by a specific PKA inhibiting peptide (Shaw et al., 1992). For the excitatory NMDA receptor channel, increases in [^{3}H]-MK-801 binding have been reported following TPA treatment (Kitamura et al., 1993) in postsynaptic densities of rat brain and sections of human spinal cord (Krieger et al., 1993). Kitamura et al. (1993) attributed these changes in [^{3}H]-MK-801 binding to changes in receptor affinity rather than number. TPA appeared to have no effect on radioligand binding to the agonist

binding site of the NMDA receptor complex, suggesting that phosphorylation only modulates channel function of NMDA receptors.

The time course required to observe changes in the amplitude of receptor-mediated currents following receptor phosphorylation appears to resemble the time course needed to detect CaMKII and PKA-induced alterations in [^{3}H]-CNQX binding. GABA$_A$ receptors expressed in human embryonic kidney 293 cells showed a 30%-40% depression of GABA currents after 15 to 20 minute treatment with 300 μM cAMP. Site-specific mutagenesis of the serine residue phosphorylated by PKA completely eliminated the depression of the GABA$_A$ receptor-mediated currents (Moss et al., 1992). In contrast, 100% increases in the amplitude of kainate receptor-mediated currents were observed after intracellular application of the catalytic subunit of PKA in human embryonic kidney 293 cells expressing GluR 6. This potentiation was complete after 35 minutes and could be blocked by a specific PKA inhibiting peptide (Raymond et al., 1993). A similar potentiation of AMPA receptor-mediated currents was reported in hippocampal neurons following treatment with activated CaMKII. This three- to four-fold increase in AMPA receptor-mediated currents was complete after approximately 12 to 15 minutes (McGlade-McCulloh et al., 1993). It is therefore possible that changes in [^{3}H]-CNQX binding reflect phosphorylation-induced changes in AMPA receptor-mediated currents. In this view, phosphorylation leads to a change in the agonist binding sites with an accompanying alteration in the receptor-mediated current response.

Although the mechanism underlying the decreases in AMPA receptor number as a result of CaMKII or PKA treatment is not clear, several possibilities can be considered: (1) receptor sequestration/ internalization, (2) receptor down-

regulation and/or (3) a change in the conformational state of the receptor resulting in decreased [^{3}H]-CNQX binding. Receptor internalization/ sequestration can be excluded, since a decrease in AMPA receptor number due to internalization of the receptors cannot be detected in freeze/thawed slices. Receptor-down regulation also did not seem to occur, since the decrease in [^{3}H]-CNQX binding was fully reversible within approximately 30 minutes, and *de novo* receptor synthesis is unlikely to occur in such a short time frame (Collins et al., 1991; Campbell et al., 1991). However, one possibility is that CaMKII and PKA lead to changes in the conformational state of the AMPA receptor agonist binding site thereby preventing binding of the labeled probe. In this case, total receptor number need not necessarily change, but <u>functional</u> receptor number will be altered. In other words, receptor proteins may still be present in the membrane, but will be maintained in a non-functional state.

Protein kinases, including CaMKII and PKA, are known to be involved in controlling the amount of neurotransmitter released presynaptically through the phosphorylation of presynaptic proteins synapsin I and II (Browning and Dudek, 1992; Greengard et al., 1993). Is it therefore possible that the observed effects of these enzymes are not attributable to receptor phosphorylation but rather to altered neurotransmitter release at the presynaptic terminal resulting in receptor regulation? This possibility seems unlikely since the present experiments were performed using a frozen/thawed preparation. This procedure should lead to a depletion of neurotransmitter due to lost membrane integrity and diffusion of neurotransmitter.

The present data reporting changes in [^{3}H]-CNQX binding in response to CaMKII activating cocktail and the catalytic subunit of PKA are in general agreement with the literature suggesting regulation of the AMPA receptor population by CaMKII (McGlade-McCulloh et al., 1993) and PKA (Keller et al., 1992; Shaw et al., 1992; Greengard et al., 1991). However, several differences remain to be resolved. McGlade-McCulloh et al. (1993) failed to see an effect of PKA on AMPA receptor phosphorylation, whereas other groups have shown AMPA receptor modification by this enzyme (Keller et al., 1992; Shaw et al., 1992; Greengard et al., 1991). How can these opposing results be reconciled? Several possible explanations can be considered. First, it has been reported that the consensus sequence for phosphorylation by CaMKII is identical to a low affinity consensus sequence for phosphorylation by PKA (Kennely and Krebs, 1991). In vitro , PKA has been shown to phosphorylate CaMKII phosphorylation sites as frequently, and perhaps as efficiently, as PKA phosphorylation sites (Kennely and Krebs, 1991). The observed effects of PKA may therefore be due to the phosphorylation of CaMKII phosphorylation sites by PKA. Second, PKA may be part of a cascade of phosphorylation reactions ultimately leading to the direct phosphorylation of AMPA receptors by CaMKII. An alternative explanation may involve differences in receptor subunit expression in the different preparations which may affect the ability of PKA to phosphorylate the AMPA receptor population. In support of this view, it has recently been reported that phosphorylation of the GABA$_A$ receptor has differential effects depending on the receptor subunit composition and the number of phosphorylation sites contained within these subunits (Krishek et al., 1994; Moss et al., 1992).

Another controversy which remains to be resolved concerns the direction of regulation of AMPA receptors observed after activation of CaMKII or treatment

with the catalytic subunit of PKA. Both CaMKII and PKA have been shown to increase AMPA receptor-mediated currents (McGlade-McCulloh et al., 1993; Keller et al. 1992; Greengard et al., 1991). However, the present results show that both of these enzymes <u>decrease</u> [^{3}H]-CNQX binding. In this regard, we have recently reported that the effects of PKA are strictly dependent on the postnatal age of the animals used (reviewed by Lanius et al., 1993; Shaw and Lanius, 1992). In animals less than 40 days postnatal age, treatment with the catalytic subunit of PKA resulted in a significant increase in [^{3}H]-CNQX binding. In contrast, in animals greater than 60 days postnatal age, PKA resulted in a significant decrease in [^{3}H]-CNQX binding. Similar results have also been reported for the GABA$_A$ receptor (reviewed by Lanius et al., 1993; Shaw and Lanius, 1992). Although the reasons for these age-dependent differences remain unknown, these results may be due to the differential expression of protein kinases (Lai and Lemke, 1991) which have been demonstrated to occur in the nervous system during development. Alternatively, differences in the expression of AMPA receptor subunits during development (Martin et al., 1993; Wenthold et al., 1992) may be contributing to the observed differences in receptor regulation. Since McGlade-McCulloh et al. (1993) and Greengard et al. (1991) used hippocampal cultures from newborn rat pups, it is conceivable that the above controversy may arise from differences in postnatal age.

Although the current data have shown that phosphorylating enzymes can alter [^{3}H]-CNQX binding, it remains unknown whether the AMPA receptor population is <u>directly</u> phosphorylated by these enzymes. Future studies using antibodies directed against GluR 1-4, phosphoserine, phosphothreonine, and phosphotyrosine residues to probe Western blots of treated and untreated rat cortical slices could resolve this issue.

In summary, the present chapter has shown that activation of endogenous CaMKII as well as treatment with the catalytic subunit of PKA can alter [^{3}H]-CNQX binding. Both treatments resulted in a decrease in [^{3}H]-CNQX binding similar to the decrease in binding observed in response to treatment with AMPA and veratridine. Moreover, the time course of regulation by CaMKII and PKA closely resembled that of AMPA and veratridine. Based on these similarities, it is possible that the regulatory action of AMPA and veratridine may be attributable to endogenous kinase activity. This issue will be explored in the chapters to follow.

CHAPTER 4

AMPA RECEPTOR REGULATION BY AGONIST AND DEPOLARIZING STIMULI REQUIRES CaMKII AND/OR PKA

Introduction

Agonist (AMPA) stimulation and pharmacologically-induced increases in cellular depolarization have been shown to lead to the regulation of cortical AMPA receptors. In cortical slices agonist stimulation as well as increases in cellular depolarization led to approximately 20% decreases in $[^3H]$-CNQX binding (Chapter 2). Using a similar *in vitro* preparation, the ability of CaMKII and PKA to induce regulation of $[^3H]$-CNQX binding was shown. Activation of CaMKII and treatment with the catalytic subunit of PKA both led to approximately 35% and 30% decreases in $[^3H]$-CNQX binding, respectively (Chapter 3).

AMPA receptor regulation by CaMKII and PKA appeared to be qualitatively similar to regulation by agonist and depolarizing stimuli. Moreover, the time course of regulation by CaMKII and PKA closely resembled the time course of AMPA- and veratridine-induced regulation. All four of these agents were able to induce maximal AMPA receptor regulation within approximately 25 min. Based on these similarities, it was hypothesized that the regulatory action of these stimuli may be attributable to endogenous kinase activity. The experiments in this chapter are therefore designed to examine whether regulation achieved by agonist and depolarizing stimuli can be blocked by selective protein kinase inhibitors. Since AMPA receptors have been shown to be regulated by CaMKII

and PKA, the effects of inhibitors of these enzymes on agonist and depolarizing stimuli will be examined.

Materials and Methods

Intact brain slices, prepared as described previously in the General Methods, were incubated for a minimum of 25 min at 37°C with a combination of the following compounds (see below) to determine their effects on AMPA receptor regulation:

A

    i) AMPA (10^{-5} M)

    ii) AMPA + CaMKII inhibitor KN-62 (10 μM)

    iii) AMPA + PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphothioate
        (Rp-cAMPS) (100 μM)

    iv) AMPA + PKC inhibitor bisindolylmaleimide (0.5 μM)

B

    i) Veratridine + Glutamate (10^{-5} M)

    i) Veratridine + Glutamate + CaMKII inhibitor KN-62 (10 μM)

    ii) Veratridine + Glutamate + PKA inhibitor Rp-cAMPS (100 μM)

    iii) Veratridine + Glutamate + PKC inhibitor bisindolylmaleimide (0.5 μM)

Following incubation with the above mentioned substances, the slices were rinsed for 2 x 30 min with 4°C DuI+. Radioligand binding was then carried out as described previously.

Inhibitor	Specificity	Concentration	Reference
KN-62	CaMKII	10 μM	1) Tansey et al., 1992 2) Ishii et al., 1991 3) Tokumitsu et al., 1990
Rp-cAMPS	PKA	100 μM	1) Wang et al., 1991 2) Van Haastert et al., 1984
Bisindolyl- maleimide	PKC	0.5 μM	Toullec et al., 1991

Table 4: Protein kinase inhibitors used to inhibit regulation by AMPA and v+g

Results

To evaluate whether the regulatory effects of AMPA on [^{3}H]-CNQX binding could be attributed to endogenous kinase activity, the effects of inhibitors of CaMKII, PKA, or PKC on decreases in [^{3}H]-CNQX binding seen as a result of AMPA receptor stimulation were examined (Figures 19&20). Treatment of cortical slices with AMPA (10^{-5} M) resulted in a significant decrease in [^{3}H]-CNQX binding to AMPA receptors (-19% to -29%) compared to control ($p<0.05$, Student's t-test) (Figure 19A n=3; Figure 19B n=4; Figure 20A n=3; Figure 20B n=3). Experiments in Figures 19A&B were not pooled since the concentration of [^{3}H]-CNQX used varied between the two experiments. The effect of AMPA could be blocked by the CaMKII inhibitor KN-62 (Figure 19A n=4; Figure 19B n=3) as well as the PKA inhibitor Rp-cAMPS (n=4) (Figure 20). The PKC

inhibitor bisindolylmaleimide was not able to prevent the decreases in [^{3}H]-CNQX binding seen as a result of AMPA receptor stimulation (n=4) (Figure 20). KN-62, bisindolylmaleimide, and Rp-cAMPS had no independent effect on [^{3}H]-CNQX binding (data not shown).

To determine whether the regulatory effects of v+g on [^{3}H]-CNQX binding could be attributed to endogenous kinase activity, the effects of inhibitors of CaMKII, PKA, or PKC on decreases in [^{3}H]-CNQX binding seen as a result of v+g treatment were examined (Figures 21&22). Treatment of cortical slices with v+g resulted in significant decreases (18%-22%) in [^{3}H]-CNQX binding compared to control (Figure 21A n=2; Figure 22A n=3; Figure 22B n=3) (p<0.05, Student's t-test) (Figures 21A & 22A&B). In Figure 21B, a one-way analysis of variance (ANOVA) showed a significant difference among the groups (p<0.05). Experiments in Figures 21A&B were not pooled since the concentration of [^{3}H]-CNQX used varied between the two experiments. The effects of v+g were abolished by the CaMKII inhibitor KN-62 (Fig. 21A n=4; Fig. 21B n=3) as well as the PKA inhibitor Rp-cAMPS (n=4). Bisindolylmaleimide was not able to inhibit the decreases in [^{3}H]-CNQX binding observed as a result of v+g treatment (n=4).

A

B

Figure 19: Regulation of AMPA receptors labeled with [3H]-CNQX. [3H]-CNQX binding was determined after agonist stimulation alone and in conjunction with selective kinase inhibitors (see Table 4). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the $p<0.05$ level (Student's t-test). Experiments were not pooled since the concentration of [3H]-CNQX used varied between the two experiments.

A

B
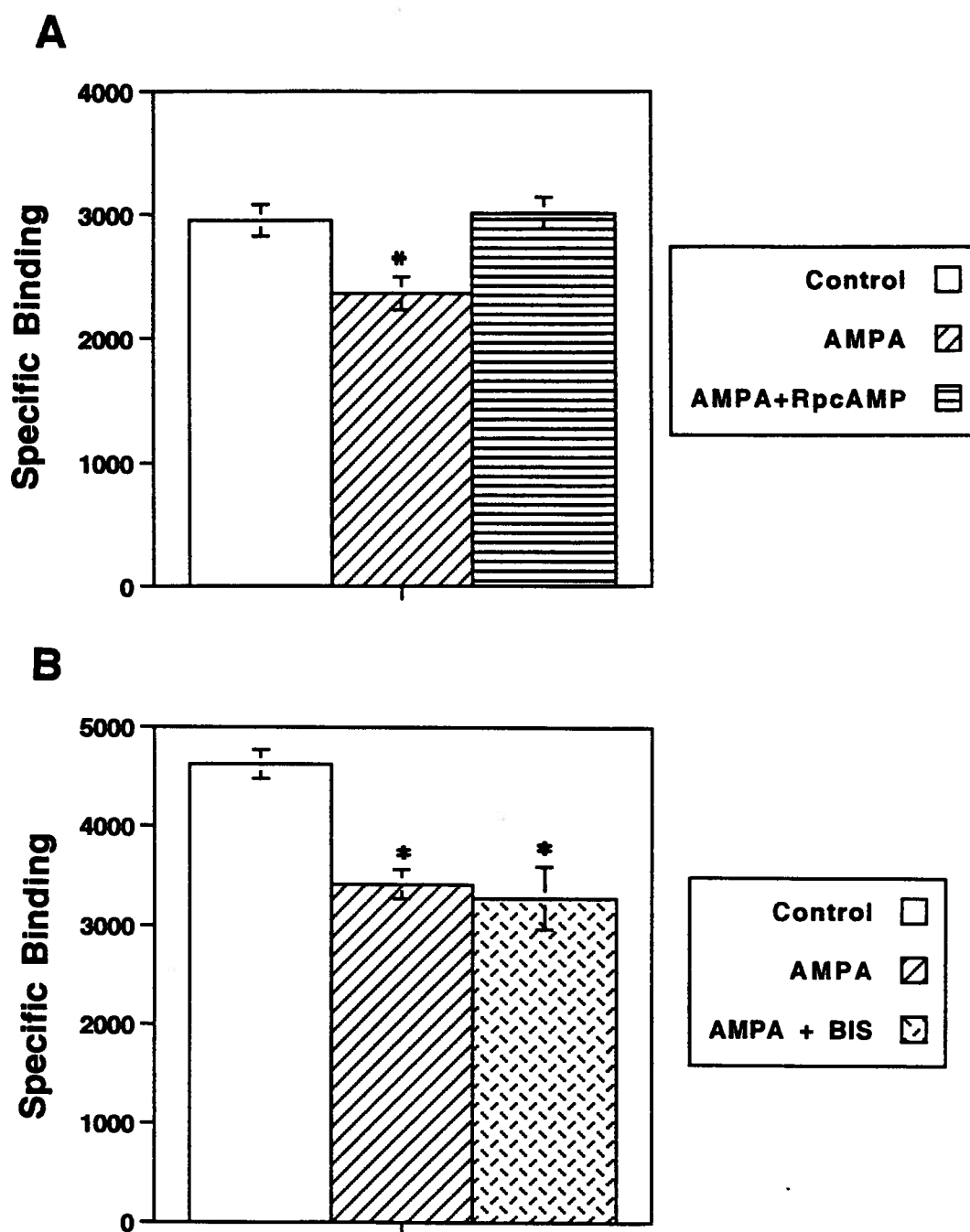
Figure 20: Regulation of AMPA receptors labeled with [^{3}H]-CNQX. [^{3}H]-CNQX binding was determined after agonist stimulation alone and in conjunction with selective kinase inhibitors (see Table 4). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the $p<0.05$ level (Student's t-test).
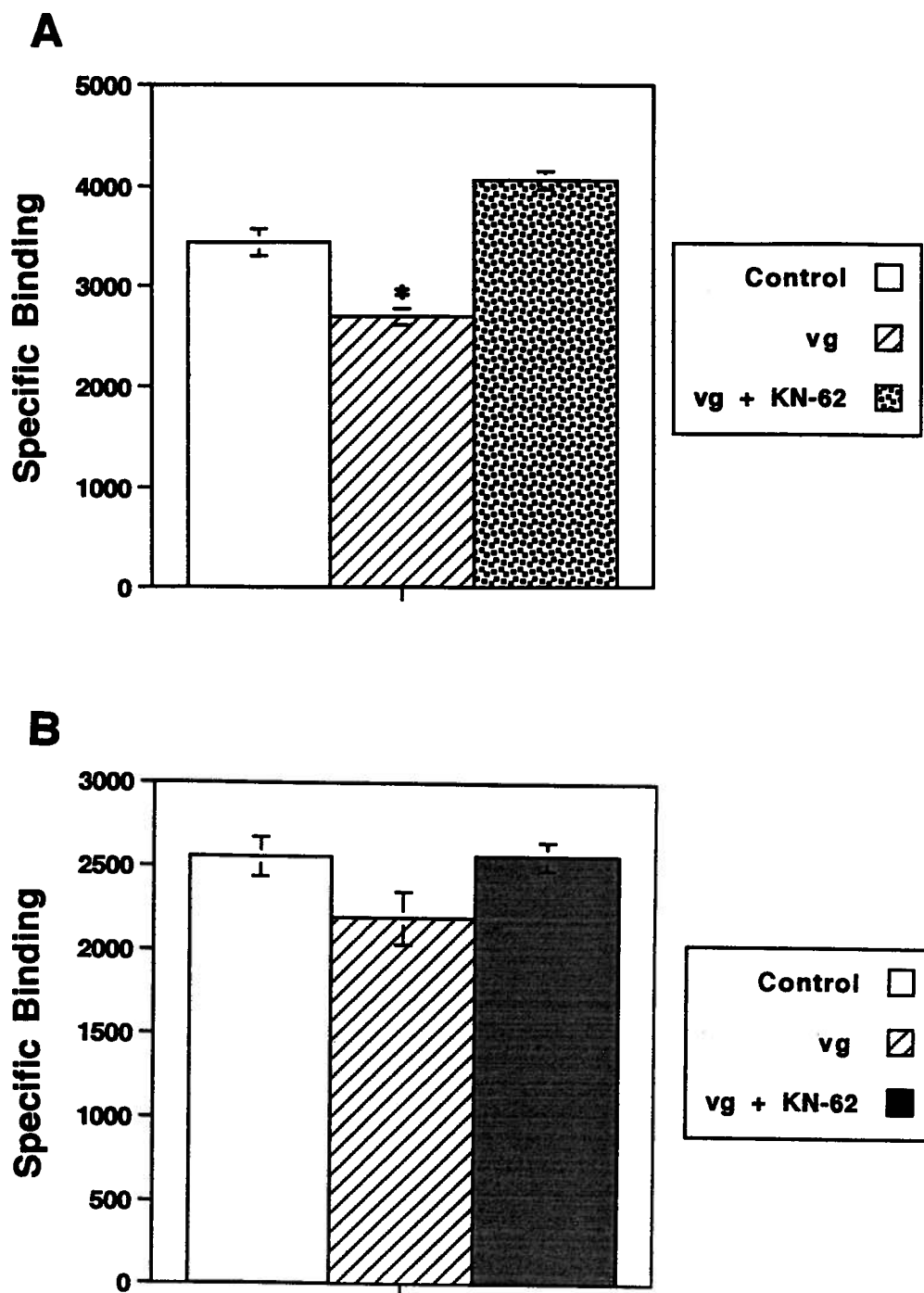
Figure 21: Regulation of AMPA receptors labeled with [^{3}H]-CNQX. [^{3}H]-CNQX binding was determined after v+g treatment alone and in conjunction with selective kinase inhibitors (see Table 4). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the $p<0.05$ level (Student's t-test). Experiments were not pooled since the concentration of [^{3}H]-CNQX used varied between the two experiments.
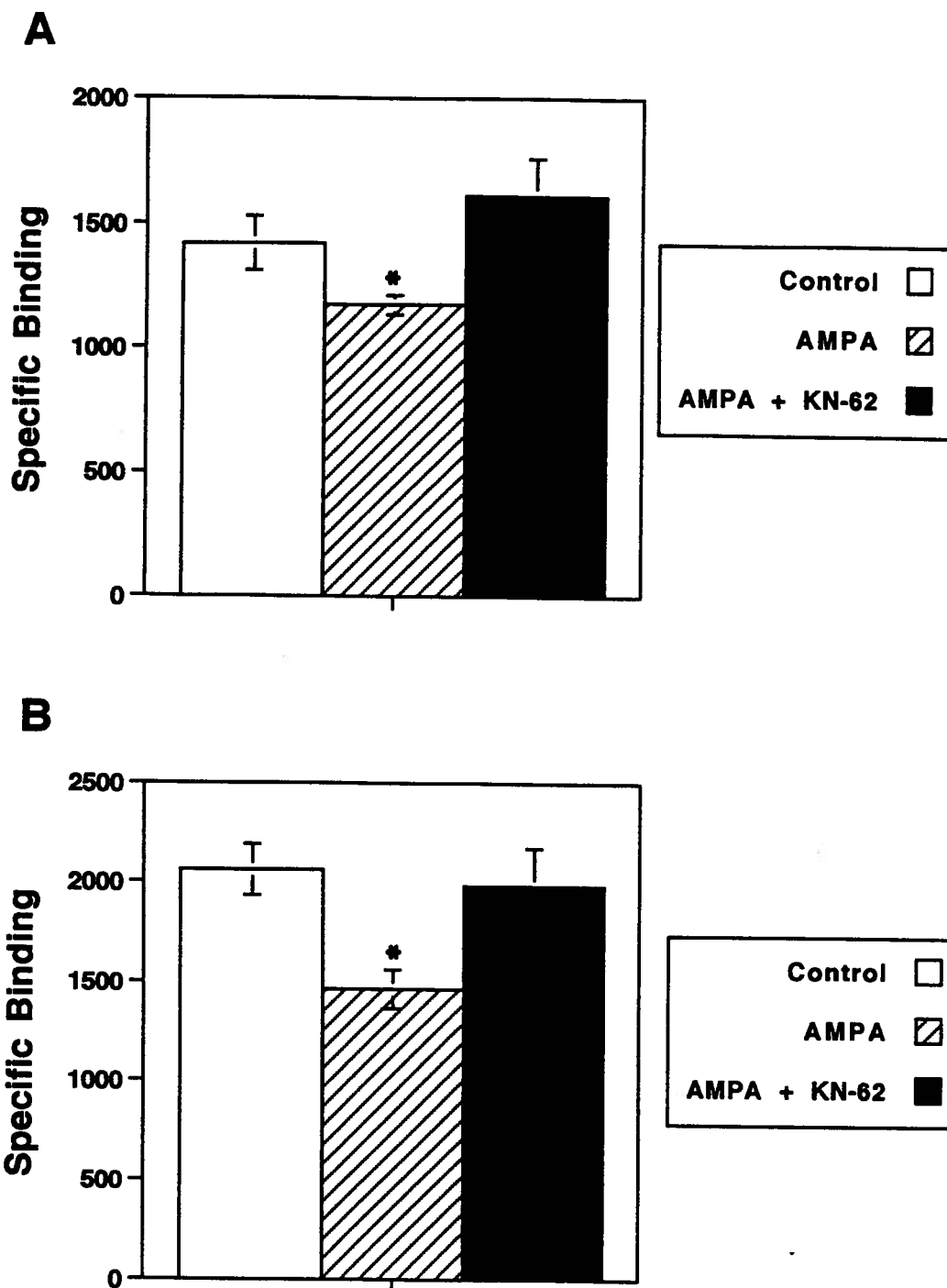
Figure 22: Regulation of AMPA receptors labeled with [³H]-CNQX. [³H]-CNQX binding was determined after v+g treatment alone and in conjunction with selective kinase inhibitors (see Table 4). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the $p<0.05$ level (Student's t-test).
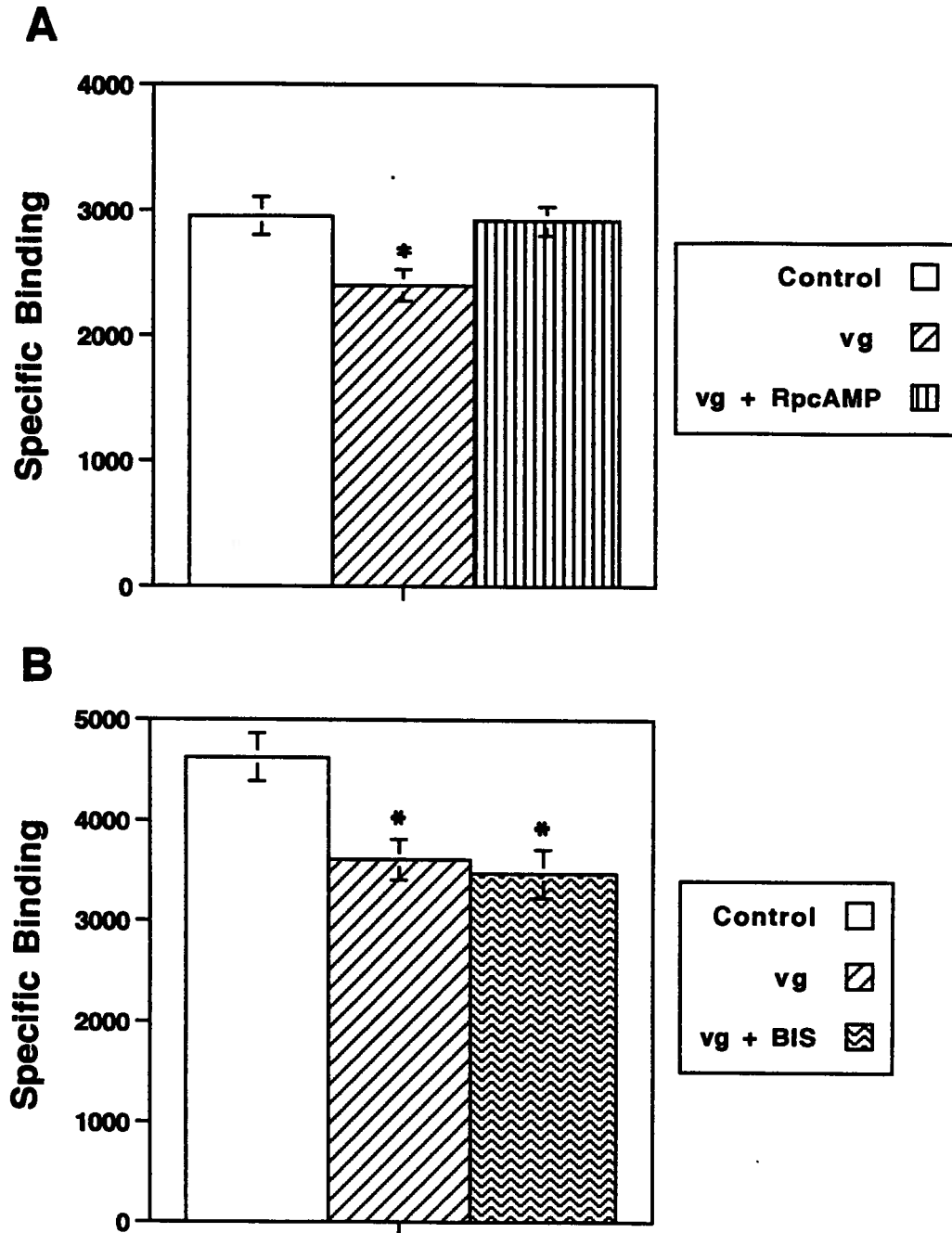
Discussion

The preceding data have shown that the decreases in [^{3}H]-CNQX binding observed as a result of AMPA or v+g treatment could be blocked by inhibitors of CaMKII and PKA (see Chapter 5 for discussion; Pasqualotto et al., 1994). The PKC inhibitor bisindolylmaleimide had no effect on AMPA receptor regulation induced by AMPA or v+g. These results suggested that protein kinases, particularly CaMKII and/or PKA, are an essential element in the regulatory response of AMPA receptors to depolarizing stimuli such as AMPA and v+g.

A common feature of both AMPA and v+g is that they cause cellular depolarization. It has previously been shown that cellular depolarization can lead to the phosphorylation of intracellular proteins. Sieghart et al. (1978) have shown that depolarization induced by veratridine and high K$^+$ stimulated the incorporation of ^{32}P into two specific proteins in a rat synaptosomal preparation. Furthermore, it is now well established that depolarization and concomitant Ca^{2+} influx into the presynaptic terminal is required for controlling the amount of neurotransmitter released through CaMK I and II- as well as PKA-mediated phosphorylation of the presynaptic proteins synapsin I and II (Greengard, 1993; Browning and Dudek, 1992). It is therefore possible that cellular depolarization induced by AMPA or v+g promotes the activation of CaMKII and/or PKA, thus resulting in decreases in [^{3}H]-CNQX binding.

It is well recognized that alterations in membrane potential result from changes in the ionic currents across the cellular membrane. Both the agonist and depolarizing stimuli employed to study activity-dependent AMPA receptor regulation are known to result in alterations in the ionic currents across the

cellular membrane. Veratridine results in increased intracellular Na^+ due to its ability to prevent the inactivation of Na^+ channels (Catterall, 1980). Indirect effects of veratridine also include increased intracellular Ca^{2+} concentrations through the depolarization-induced activation of voltage-gated Ca^{2+} channels. Activation of cortical AMPA receptors leads to cellular depolarization due to increased Na^+ fluxes through AMPA receptors (Seeburg, 1993) with concomitant increases in intracellular Ca^{2+} due to the depolarization-induced activation of voltage-gated Ca^{2+} channels (Church et al., 1994). The role of these ions in mediating decreases in $[^3H]$-CNQX binding will therefore be the focus of the following chapter.

CHAPTER 5

Ca^{2+}-DEPENDENCE OF AMPA RECEPTOR REGULATION

Introduction

A common feature of both agonist and depolarizing stimuli employed to modulate AMPA receptor characteristics is that they cause transient, but profound changes in postsynaptic ionic concentrations within the cell. Changes in the postsynaptic intracellular ionic environment as a result of agonist or depolarizing stimuli may provide the trigger for the control of AMPA receptor regulation by phosphorylation. In this view, the ionic currents which cross neural membranes in response to various depolarizing stimuli will activate specific kinases leading to receptor regulation. For AMPA receptors, whose activation by agonists leads to a direct influx of Na^+ as well as a secondary influx of Ca^{2+} through voltage-gated Ca^{2+} channels, a role for one or both of these ions in the activation of kinase-mediated decreases in $[^3H]$-CNQX binding will be investigated. Experiments will examine the effects of Ca^{2+} and Na^+ on $[^3H]$-CNQX binding. Moreover, to examine a potential role for voltage-gated Ca^{2+} channels in AMPA receptor regulation by agonist and depolarizing stimuli, the effects of loperamide, a non-specific blocker of voltage-gated Ca^{2+} channels, will be investigated. Electrophysiological studies have recently shown that Ca^{2+} influxes through voltage-gated Ca^{2+} channels as a result of AMPA receptor stimulation could be blocked by the antidiarrheal agent loperamide (Church et al., 1994).

Although the effects of Ca^{2+} as a trigger for certain kinases such as Ca^{2+}-phospholipid-dependent PKC (Nishizuka, 1988) and Ca^{2+}/calmodulin-dependent kinase (Hanson and Schulman, 1992) have been well documented, much earlier studies had shown that the phosphorylation of certain membrane proteins of the electric organs of *Torpedo Californica* and *Torpedo mamorata* were controlled by specific ionic species (Na^+ and K^+) not usually associated with the activation of protein kinases (Gordon et al., 1977; Saitoh and Changeux, 1980). Saitoh and Changeux (1980) reported that an increase in the concentration of Na^+ from 25 to 250 mM led to a decrease of the phosphorylation of approximately 80% of membrane proteins in *Torpedo mamorata* ; increasing concentrations of K^+ enhanced the phosphorylation of some polypeptide chains with a molecular weight equal to or higher than 85 000. Similarly, Gordon et al. (1977) showed that phosphorylation of several polypeptides from the electric organ of *Torpedo californica* was stimulated by the addition of K^+. These results show that although Ca^{2+} appears to be the most likely candidate for triggering phosphorylation reactions, a role for other ions (Na^+/K^+) cannot be ruled out.

Materials and Methods

The Effects of Ca^{2+}, Na^+, and K^+ on [^{3}H]-CNQX Binding

Full details of the cortical slice preparation and receptor assays are discussed in the General Methods. The brains of Sprague-Dawley rats were rapidly removed and dissected in a 50 mM Tris-acetate buffer (pH 7.4). The slices obtained from each brain were placed into tissue culture wells, slowly frozen to -30°C, and then rapidly thawed.

Slices were then pre-incubated in different concentrations of sodium acetate (6-200 mM), potassium acetate (6-200 mM), or calcium acetate (0.1-1 mM) for a minimum of 25 min at 37°C before incubation with [^{3}H]-CNQX. Similar Ca^{2+} concentrations have been previously employed to activate CaMKII (Tan et al., 1994; McGlade-McCulloh et al., 1993). The buffers were adjusted to physiological pH (7.4) using 99% glacial acetic acid in all cases.

In order to determine whether the effects observed were due to ions acting on potential endogenous kinases, slices were co-incubated with the specific ions in addition to selective kinase inhibitors: 10 μM KN-62, a CaMKII specific inhibitor (Tokumitsu et al., 1990), 5 μM protein kinase A inhibiting peptide (Smith et al., 1990), or 0.5 μM bisindolylmaleimide, a PKC inhibitor (Toullec et al., 1991).

Following the preincubation step, slices were rinsed for 2 x 30 min with 100 mM Tris-acetate buffer (4°C) and incubated with 5-10 nM [^{3}H]-CNQX in Tris-acetate buffer for 3 h at 4°C. After the radioligand binding step, slices were rinsed for 2 x 5 min with 100 mM Tris-acetate buffer (pH 7.4) at 4°C and counted in a Beckman 6000 IC scintillation counter.

The Effects of Loperamide on AMPA Receptor Regulation by Agonist and Depolarizing Stimuli

Intact brain slices were prepared as described in the General Methods. Slices were co-incubated with AMPA (10^{-5} M) or v+g (10^{-5} M) and 25 μM or 100 μM loperamide (Church et al., 1994; Pasqualotto et al., 1994) in Dul^+ for a minimum of 25 min at 37°C. Following this incubation, slices were rinsed for 2 x 30 min

with DuI+ at 4°C. Radioligand binding was then carried out as described in the General Methods.

<u>Results</u>

<u>The Effects of Ca^{2+}, Na$^+$, and K$^+$ on [^{3}H]-CNQX Binding</u>

To determine the effects of Ca^{2+} on [^{3}H]-CNQX binding, the effects of Ca^{2+} (0.1-1 mM) alone and in combination with KN-62, PKAIP, or bisindolylmaleimide were examined (Figures 23 & 24). Ca^{2+} significantly decreased [^{3}H]-CNQX binding over a concentration range of 0.1 to 1 mM (p<0.05, Student's t-test). This decrease could be partially blocked by PKAIP (n=3). KN-62 was able to completely inhibit Ca^{2+}-induced decreases in [^{3}H]-CNQX binding (n=3). Bisindolylmaleimide had no effect on the decrease in [^{3}H]-CNQX binding observed after incubation with Ca^{2+} (n=3). Neither KN-62, PKAIP, nor bisindolylmaleimide had any independent effect on [^{3}H]-CNQX binding (data not shown).

The effects of Na$^+$ and K$^+$ (6-200 mM) on [^{3}H]-CNQX binding were examined (Figure 25). Neither K$^+$ nor Na$^+$ in this concentration range had a significant effect on [^{3}H]-CNQX binding.

In order to control for possible effects of acetate alone, the effect of calcium acetate was compared to that of sodium and potassium acetate. Calcium acetate resulted in a decrease in [^{3}H]-CNQX binding, whereas neither sodium nor potassium showed an effect.

The Effects of Loperamide on AMPA Receptor Regulation by Agonist and Depolarizing Stimuli

To establish whether AMPA-induced regulation of AMPA receptors requires an influx of Ca^{2+} through voltage-gated Ca^{2+} channels, the effects of loperamide (25 μM or 100 μM) on decreases in $[^3H]$-CNQX binding seen as a result of AMPA treatment were examined (Figure 26). Treatment with 10^{-5} M AMPA resulted in a significant decrease in $[^3H]$-CNQX binding ($p<0.05$, Student's t-test) which could only be partially blocked by 25 μM loperamide (n=5) (Figure 26A). However, the decrease in $[^3H]$-CNQX binding as a result of AMPA treatment could be completely blocked by 100 μM loperamide (n=5) (Figure 26B). A one-way analysis of variance (ANOVA) showed a significant difference among the groups ($p<0.05$). Loperamide did not have any significant independent effects on $[^3H]$-CNQX binding (data not shown).

To determine whether veratridine-induced regulation of AMPA receptors requires an influx of Ca^{2+} via voltage-gated Ca^{2+} channels, the effects of loperamide (100 μM) on decreases in $[^3H]$-CNQX binding seen as a result of v+g treatment were examined (Figure 27). Treatment with veratridine resulted in significant 23% decreases in $[^3H]$-CNQX binding ($p<0.05$, Student's t-test) which could be completely blocked by 100 μM loperamide (n=3).
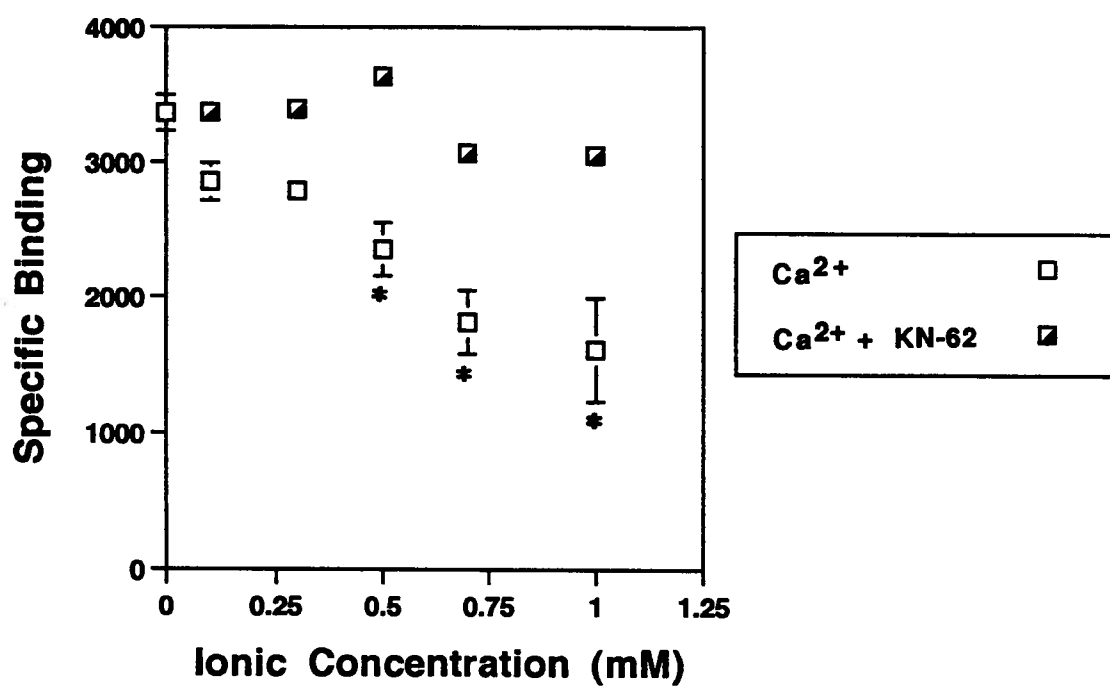
Figure 23: Effects of Ca^{2+} on [^{3}H]-CNQX Binding. Significance to the p<0.05 level (Student's t-test) is indicated by an asterisk. Error bars show S.E.M. (see Results for n values). Specific binding is expressed as dpm.

A
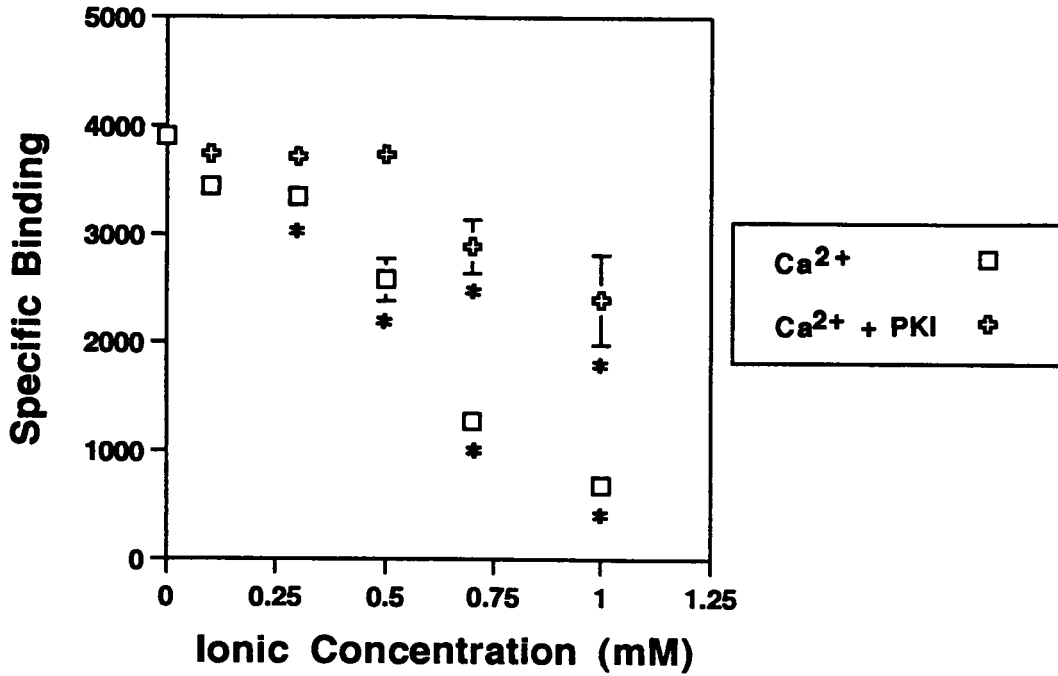
B
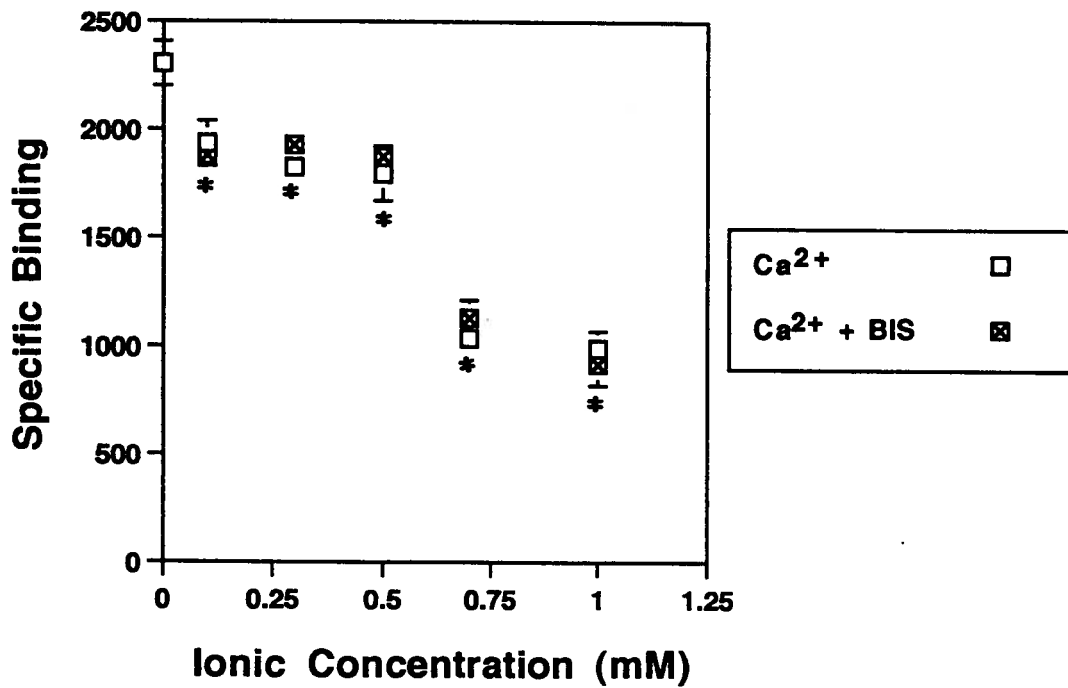
Figure 24: Effects of Ca^{2+} on [^{3}H]-CNQX Binding. Significance to the p<0.05 level (Student's t-test) is indicated by an asterisk. Error bars show S.E.M. (see Results for n values). Specific binding is expressed as dpm.

A

B
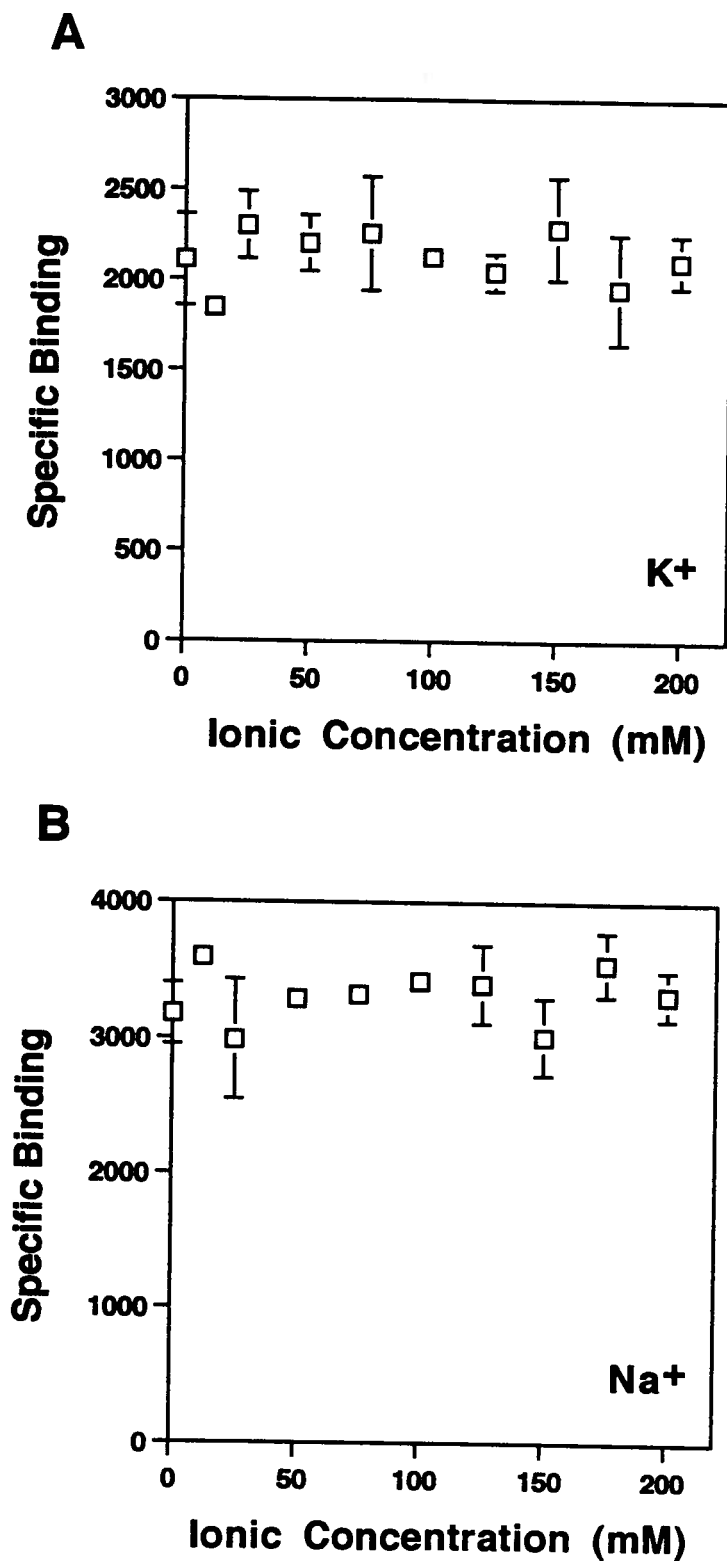
Figure 25: Effects of K+ and Na+ on [3H]-CNQX Binding. Error bars show

S.E.M. (see Results for n values). Specific binding is expressed as dpm.

A

B
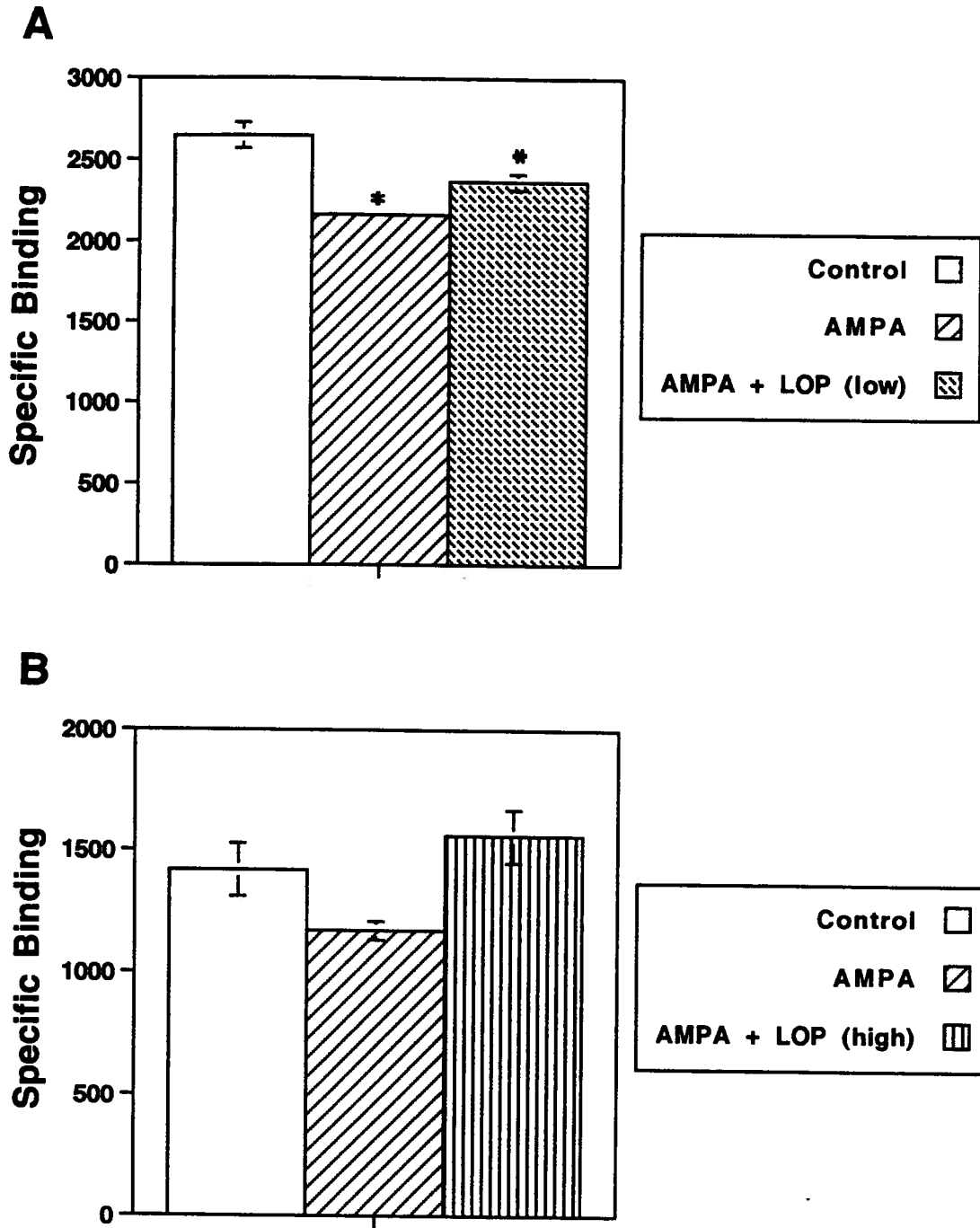
Figure 26: The effects of loperamide on AMPA receptor regulation by AMPA. [^{3}H]-CNQX binding was determined after AMPA treatment alone and in conjunction with loperamide (25 μM or 100 μM). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the p<0.05 level (Student's t-test). Specific binding is expressed as dpm.
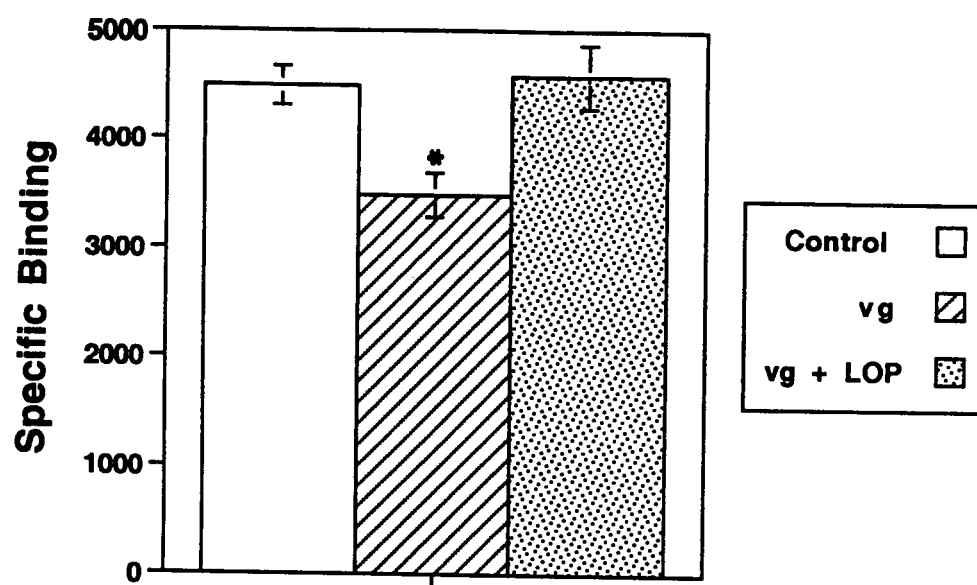
Figure 27: The effects of loperamide on AMPA receptor regulation by v+g. [^{3}H]-CNQX binding was determined after v+g treatment alone and in conjunction with loperamide (100 μM). Error bars show S.E.M. (see Results for n values), and asterisks indicate significance to the p<0.05 level (Student's t-test). Specific binding is expressed as dpm.

Discussion

A decrease in [^{3}H]-CNQX binding was observed in response to treatment with
0.1-1 mM calcium acetate. These decreases in binding could be completely
blocked by the CaMKII inhibitor KN-62 and partially blocked by a PKA inhibiting
peptide. Nielsen et al. (1990) have previously examined the effects of 2.5 mM
calcium acetate on [^{3}H]-CNQX and [^{3}H]-AMPA binding in cerebellar rat brain
sections. Although they reported a significant decrease in [^{3}H]-AMPA binding
in response to treatment with 2.5 mM calcium acetate, the effect of calcium
acetate on [^{3}H]-CNQX (50 nM) binding was not statistically significant.
However, since 50 nM [^{3}H]-CNQX labels AMPA- as well as kainate receptors, it
is possible that Ca^{2+}-mediated effects on the AMPA receptor population are
masked by [^{3}H]-CNQX binding to kainate receptors.

Much earlier studies had shown the ability of Ca^{2+} to modify [^{3}H]-glutamate
binding in rat hippocampus through an unknown mechanism (Baudry and
Lynch, 1979). Ca^{2+} was found to be very potent in enhancing [^{3}H]-glutamate
binding over a concentration range of 25 μM to 50 mM. However, maximal
effects (100%) were seen at a concentration of approximately 1 mM. Scatchard
analyses of [^{3}H]-glutamate binding in the presence of 1 mM Ca^{2+} suggested
that Ca^{2+} altered the maximum number of [^{3}H]-glutamate binding sites without
changing their affinity.

Since Ca^{2+} led to decreases in [^{3}H]-CNQX binding which could be blocked by
the CaMKII inhibitor KN-62, the role of Ca^{2+} in AMPA receptor regulation by
AMPA and veratridine were further examined. Loperamide, a non-specific
blocker of voltage-gated Ca^{2+} channels, was able to inhibit the decrease in

[^{3}H]-CNQX binding observed in response to treatment with AMPA or v+g (Pasqualotto et al., 1994). Loperamide has previously been shown to block Ca^{2+} influxes through voltage-gated Ca^{2+} channels as a result of AMPA receptor stimulation in hippocampal pyramidal neurons (Church et al., 1994). These results suggested that Ca^{2+} influx through voltage-gated Ca^{2+} channels is necessary for AMPA- and veratridine-induced AMPA receptor regulation via CaMKII- and/or PKA-mediated phosphorylation reactions. However, loperamide has also been shown to display weak calmodulin antagonist activity (IC_{50} values ranging from 75-100 μM) (Kachur et al., 1986). It is therefore plausible that the inhibitory effects of this compound are due to calmodulin antagonism. Experiments employing other Ca^{2+} channel blockers, including nifedipine (L-type channel blocker) and ω-conotoxin (N-type channel blocker) could distinguish between calmodulin- and Ca^{2+} channel-mediated antagonism.

It remains unclear why only the higher concentration of loperamide (100 μM) was able to block the effects of AMPA. However, it is possible that this concentration was more effective since it blocks both voltage-gated Ca^{2+} channels and calmodulin.

How do AMPA and veratridine fulfill the requirements for the activation of CaMKII and PKA? The factors leading to the activation of CaMKII, PKA and PKC have been well documented. CaMKII relies on the presence of both Ca^{2+} and calmodulin for its activation. In the presence of micromolar concentrations of Ca^{2+}, calmodulin undergoes a conformational change exposing hydrophobic binding sites which interact with a calmodulin-binding domain on the enzyme. Binding of Ca^{2+}/calmodulin is thought to unfold the molecule, thereby exposing

and activating the catalytic region (for review see Fujisawa, 1992; Hanson and Schulman, 1992).

In contrast to CaMKII, the activation of PKA is dependent on the production of cAMP which usually results from the stimulation of adenylate cyclase following the activation of certain G-protein coupled receptors (for review Scott, 1991; Nairn et al., 1985). In the absence of cAMP, the enzyme consists of two regulatory subunits bound to two catalytic subunits. The binding of cAMP to the regulatory subunits leads to the dissociation of the catalytic subunits, which are now able to express phosphotransferase activity.

Since agonist (AMPA) stimulation as well as chemically-induced increases in depolarization can lead to an increase in intracellular Ca^{2+} concentrations, such treatments could lead to the activation of CaMKII with concomitant changes in [^{3}H]-CNQX binding. An alternative mechanism of CaMKII activation has been suggested by Fukanaga et al. (1992) and Tan et al. (1994) who reported activation of CaMKII in response to increases in intracellular Ca^{2+} as a result of NMDA receptor activation in cultured hippocampal neurons. Glutamate elevated the Ca^{2+}-independent activity of CaMKII, an effect which could be blocked by the NMDA receptor antagonist AP-5.

Although increases in intracellular Ca^{2+} (reviewed by Nishizuka, 1988) as a result of agonist stimulation or increases in cellular depolarization induced by veratridine could lead to the activation of PKC, the PKC inhibitor bisindolylmaleimide had no effect on the agonist- or veratridine-induced decreases in [^{3}H]-CNQX binding. These results are not surprising considering the lack of effect of TPA on [^{3}H]-CNQX binding (Chapter 3).

It remains unclear why the inhibiting peptide of a cAMP-dependent enzyme (PKA) and RpcAMPS would block Ca^{2+}-mediated decreases in [^{3}H]-CNQX binding. These results are especially unexpected since Ca^{2+} can lead to the activation of phosphodiesterase, an enzyme which breaks down cAMP (Stryer, 1988). However, several possibilities seem plausible:

(1) It has been reported that the consensus sequence for phosphorylation by CaMKII is identical to a low affinity consensus sequence for phosphorylation by PKA (Kennely and Krebs, 1991). It is therefore possible that the effects of the PKA inhibiting peptide, an inhibitor whose mechanism is based on the consensus sequence of PKA, are due to the partial inhibition of CaMKII. Moreover, RpcAMPS at the concentration used in the present experiments (0.1 mM) has been shown to bind competitively to the ATP binding site of protein kinases (Pelech, personal communication) and may therefore act to inhibit CaMKII activity and associated decreases in [^{3}H]-CNQX binding.

(2) Agonist and depolarizing stimuli activate both PKA and CaMKII through an unknown mechanism, leading to AMPA receptor regulation through phosphorylation by both PKA and CaMKII. This mechanism, however, is unlikely to occur since both PKA and CamKII inhibitors are able to independently block the effects of agonist and depolarizing stimuli.

(3) Activation of AMPA receptors results in a Ca^{2+} influx via voltage-gated Ca^{2+} channels leading to the activation of CaMKII. Activation of the latter represents the initial step in a cascade of phosphorylation reactions ultimately leading to the direct phosphorylation of AMPA receptors by PKA. However, this possibility seems unlikely for two reasons. First, AMPA receptors (GluR 1 - GluR 4) do not appear to have consensus sequences for phosphorylation by PKA (Keinanen et

al., 1990; Boulter et al., 1990). Second, PKA does not appear to directly phosphorylate AMPA receptors (Tan et al., 1994; McGlade-McCulloh et al., 1993).

(4) Activation of AMPA receptors results in a Ca^{2+} influx through voltage-gated Ca^{2+} channels, leading to the activation of PKA through the activation of adenylyl cyclase. In regard to the latter, it has recently been shown that activation of NMDA receptors results in an increase in cAMP via Ca^{2+}/calmodulin stimulation of adenylyl cyclase (Chetkovich and Sweatt, 1993). The activation of PKA, in turn, results in a cascade of phosphorylation reactions, ultimately leading to the direct phosphorylation of AMPA receptors by CaMKII. This option appears more likely at present due to the presence of strong consensus sequences for phosphorylation by CaMKII on GluR 1-4 as well as the ability of CaMKII to directly phosphorylate the AMPA receptor (McGlade-McCulloh et al., 1993).

(5) An alternative explanation would be that activation of AMPA receptors results in an influx of Ca^{2+} via voltage-gated Ca^{2+} channels resulting in the activation of both PKA and CaMKII through an unknown mechanism. The latter are both required for the activation of an unidentified AMPA receptor specific kinase, leading to the direct phosphorylation of the AMPA receptor population.

A receptor specific kinase proposed above would be similar to the specific receptor kinase described for the adrenergic receptors (Benovic et al., 1987) and postulated for the GABA$_A$ receptor population (Sweetnam et al., 1988). The ß-adrenergic kinase (ßark) is a cAMP-independent kinase that specifically phosphorylates the agonist-occupied forms of the β_2-adrenergic receptor.

Receptor phosphorylation by ßark promotes the binding of another protein, ß-arrestin, and this interaction appears to result in uncoupling of receptors and the associated G-protein (Benovic et al., 1987). Evidence for a novel $GABA_A$ receptor-associated kinase comes from results which showed $GABA_A$ receptor phosphorylation by a receptor-associated protein kinase in partially purified preparations of $GABA_A$ receptors (Sweetnam et al., 1988). This novel, second-messenger-independent protein kinase may be involved in the functional modification of $GABA_A$ receptors.

Although the exact order and constituents of the cascade of events leading to AMPA receptor regulation by agonist and depolarizing stimuli remain unknown, PKA and/or CaMKII seem to be involved in achieving such regulation. The involvement of multiple kinases in AMPA receptor regulation does not appear to be unique to this receptor population. For example, as discussed in the General Introduction, an examination of the amino acid sequence of $GABA_A$ and nACh receptor subunits revealed the existence of consensus sequences for a variety of protein kinases, including PKA, PKC, and a protein tyrosine kinase (Swope et al., 1992). For $GABA_A$ receptors, phosphorylation by any one of these kinases resulted in a decrease in $GABA_A$ receptor-mediated currents (Moss et al., 1992). Similarly, for nACh receptors, phosphorylation by PKA, PKC, or a protein tyrosine kinase led to an increase in the rapid rate of desensitization (Hopfield et al., 1988; Huganir et al., 1986). Phosphorylation of $GABA_A$ and nACh receptors can thus be regulated by several second messenger systems, allowing for the heterologous regulation of these receptor populations. Although AMPA receptors also show consensus sequences for a variety of protein kinases, the regulation of this receptor population by agonist

and depolarizing stimuli may be different in that it requires more than one protein kinase.

There are two major implications of the present results. First, the data provide further evidence for the involvement of Ca^{2+} in the regulation of AMPA receptors by phosphorylation. Although it remains unknown whether Ca^{2+} directly activates the kinase(s) involved in AMPA receptor regulation, such a process may reveal a crucial step in the cascade of events leading to AMPA receptor regulation. Such a cascade may begin with neurotransmitter binding to AMPA receptors leading to an alteration in ionic current through channels associated with the receptors and voltage-gated Ca^{2+} channels with subsequent changes in intracellular Ca^{2+} concentrations. Changes in intracellular Ca^{2+} concentrations may then be the factors triggering specific kinase(s), ultimately leading to the regulation of the receptors by phosphorylation.

At a practical level, the above results suggest that the medium in which receptor binding studies are carried out can alter radioligand binding by stimulation of endogenous kinases. These findings make it imperative to strictly control the conditions under which such binding studies are performed. Attempts to modify the media by the additions of various ions might inadvertently lead to ion-mediated enzyme action and result in changes in binding levels. The likelihood that such variations will occur necessitates a careful examination of ionic-dependence in each separate case.

GENERAL DISCUSSION

Summary of Data

(1) A [^{3}H]-CNQX binding site in rat cortex was studied and found to satisfy the basic criteria for receptor characterization (Boulton et al., 1985), including steady-state binding, competition by specific AMPA analogues, as well as saturable binding. These data allowed the description of this [^{3}H]-CNQX binding site as an AMPA receptor (Chapter 1).

(2) AMPA or v+g treatment of cortical slices resulted in reversible decreases in [^{3}H]-CNQX binding (Chapter 2).

(3) Activation of endogenous CaMKII and treatment with the catalytic subunit of PKA also led to reversible decreases in [^{3}H]-CNQX binding. These enzyme-induced alterations in [^{3}H]-CNQX binding resembled the decreases in [^{3}H]-CNQX binding observed in response to AMPA and v+g treatment, both qualitatively and quantitatively (Chapter 3).

(4) AMPA receptor regulation induced by AMPA and v+g could be completely blocked by CaMKII and PKA inhibitors. Loperamide, a non-specific inhibitor of voltage-gated Ca^{2+} channels, was also able to abolish the regulatory effects of these stimuli. These results suggested that CaMKII, PKA, as well as an influx of Ca^{2+} through voltage-gated Ca^{2+} channels are involved in the regulation of AMPA receptors by agonist and depolarizing stimuli (Chapter 4).

(5) Calcium ions alone were sufficient to produce significant, concentration-dependent decreases in [^{3}H]-CNQX binding over a concentration range of 0.1-1 mM. These decreases in binding could be blocked by CaMKII and PKA inhibitors. Although it remains unclear why the inhibiting peptide of a cAMP-dependent enzyme (PKA) would block Ca^{2+}-mediated decreases in [^{3}H]-CNQX binding, several possibilities are discussed in Chapter 5.

These data have provided direct measurements of alterations in the agonist binding site of the AMPA receptor. Although cellular current responses following activity-dependent regulation of AMPA receptors have not been evaluated, it is probable that a decrease in [^{3}H]-CNQX binding reflects a diminution of AMPA receptor-mediated currents. Decreases in AMPA receptor number and/or affinity should offer fewer targets to endogenous neurotransmitter, leading to a decrease in postsynaptic currents to the same level of presynaptic stimulation. With regard to the latter, Shahi and Baudry (1992) have reported that increasing binding affinity of agonists to AMPA receptors increases synaptic responses at glutamatergic synapses.

A Model of AMPA Receptor Regulation

The results described above suggest a cascade of events leading to AMPA receptor regulation. Such a cascade may begin with cellular depolarization through ion channels associated with the AMPA receptor population and/or voltage-gated ion channels and subsequent changes in intracellular Ca^{2+} concentrations. Changes in subsynaptic Ca^{2+} concentrations may then be the factors triggering specific kinases, ultimately leading to AMPA receptor regulation by phosphorylation. Alterations in receptor binding may, in turn, alter

the neural response to subsequent neurotransmitter release. A cascade of this type proposed here may prove to be a general mechanism underlying ionotropic receptor regulation.

A heterologous mechanism may also be proposed in which regulation of AMPA receptors occurs through the activation of a G-protein coupled or another ionotropic receptor population. Activation of a G-protein coupled receptor population may lead to the production of cAMP in turn resulting in the activation of PKA and concomitant regulation of AMPA receptors. Alternatively, activation of another ionotropic receptor population (e.g. NMDA) may lead to an alteration in ionic current through receptor associated ion channels, or, alternatively, through the activation of voltage-gated ion channels.

AMPA Receptor Regulation: Implications for Neural Function

The consequences of AMPA receptor regulation may have important implications for many aspects of neural function. First, alterations in functional AMPA receptor levels may play an integral role in the control of normal synaptic neurotransmission. Second, AMPA receptor regulation may be a key component for the maintenance of some forms of synaptic neuroplasticity. Finally, AMPA receptor regulation in response to a neurotoxic environment may reduce the neurotoxic damage and cell death associated with certain neurodegenerative disorders.

AMPA Receptor Regulation in Normal Synaptic Neurotransmission

The regulation of AMPA, and other ionotropic receptors, in response to depolarizing stimuli may be a feature in the control of normal synaptic transmission. Alterations in functional receptor levels and responses may determine the level of response to subsequent neurotransmitter release. This, in turn, may account for transient changes in the efficacy of synaptic neurotransmission and could constitute a possible homeostatic mechanism in the control of interneuronal communication.

AMPA Receptor Regulation in Synaptic Neuroplasticity

Depolarizing stimuli may, under certain conditions, result in long-lasting alterations in functional AMPA receptor levels, thus resulting in a permanent alteration of the level of response to subsequent neurotransmitter release. Changes in AMPA receptor number have been postulated to provide the basis for some forms of synaptic plasticity, including LTP (Maren et al., 1993; Tocco et al., 1992) and cerebellar LTD (Linden, 1994). The stages in AMPA receptor regulation may provide more details concerning such phenomena. Thus the present model would predict that for plastic modifications of function to occur AMPA receptors controlling particular ionic currents must be present. Second, regulatory enzymes, e.g. kinases, must be present. The action of such enzymes will, in some cases, be controlled by specific ions and ionic concentrations. Ionic currents following neurotransmitter activation of receptors will, in turn, act to turn on regulatory enzymes which will then lead to receptor regulation by phosphorylation. The modification in [^{3}H]-CNQX binding in such cases may provide for a LTD-like phenomenon similar to the one observed between

parallel fibres and Purkinje cells in the cerebellum (reviewed by Linden et al., 1994) (see General Introduction). LTD is thought to be mediated entirely by a decrease in the number or sensitivity of postsynaptic AMPA receptors as a result of protein kinase activity (Linden, 1994; Linden et al., 1993; 1991).

Receptor Regulation and Neuropathology

In many neurologic disorders, including ischemia, epilepsy, as well as more chronic neurodegenerative states such as amyotrophic lateral sclerosis, neurolathyrism, and Huntington's disease, neuronal damage has been postulated to arise through an overstimulation of excitatory amino acid receptors by glutamate or aspartate (reviewed by Lipton and Rosenberg, 1994). Overstimulation of glutamate receptors results in increased intracellular Ca^{2+} levels which, in turn, have been shown to lead to cell death through the activation of proteases, nitric oxide synthase, as well as PKC and CaMKII (reviewed by Lipton and Rosenberg, 1994).

A decrease in $[^3H]$-CNQX binding in response to an overstimulation of AMPA or other ionotropic receptors may be viewed as a neuroprotective mechanism. Decreases in functional AMPA receptor binding should potentially lead to decreased Ca^{2+} influx and associated CaMKII activity. Moreover, it may prove possible to deliberately manipulate elements in the AMPA receptor regulation cascade in order to control increased Ca^{2+} influx and CaMKII activation, thus reducing neurotoxic damage and cell death associated with certain neurodegenerative disorders.

Concluding Remarks

In conclusion, AMPA receptor regulation may be involved in many aspects of neuronal function, including normal synaptic neurotransmission, synaptic neuroplasticity, and some forms of neuropathology. A better understanding of the molecular mechanisms involved in the regulation of AMPA receptors and ionotropic receptors in general may provide a framework for studies of synaptic function and dysfunction.

FUTURE DIRECTIONS

(1) Electrophysiological experiments examining AMPA receptor-mediated currents in response to treatment with AMPA, v+g, or activators of CaMKII and PKA could be carried out to determine functional correlates of decreases in $[^3H]$-CNQX binding. These experiments would allow a direct comparison between changes in $[^3H]$-CNQX binding and alterations in AMPA receptor-mediated current responses.

(2) To determine whether treatment with AMPA, v+g, an activating cocktail of CaMKII, or the catalytic subunit of PKA leads to a change in the phosphorylation state of the AMPA receptor population, studies using antibodies directed against GluR 1-4, phosphoserine, phosphothreonine, and phosphotyrosine residues to probe Western blots of treated and untreated rat cortical slices could be carried out. These experiments would determine whether changes in the phosphorylation state of AMPA receptors correlate with changes in $[^3H]$-CNQX binding.

(3) CaMKII and PKA activity assays could be carried out to allow the determination of CaMKII and PKA phosphotransferase activity as a result of AMPA and veratridine treatment. Furthermore, CaMKII and PKA phosphotransferase activity could be measured following treatment with AMPA or v+g and inhibitors of CaMKII, PKA, and voltage-gated Ca^{2+} channels. Addition of these inhibitors should abolish any increase in CaMKII and PKA phosphotransferase activity induced by AMPA or v+g.

(4) To further investigate the role of Ca^{2+} and voltage-gated Ca^{2+} channels in AMPA receptor regulation, AMPA- and v+g-induced receptor regulation could be studied in a Ca^{2+}-free medium. The inability of AMPA and v+g to induce AMPA receptor regulation in such a medium would provide further evidence that the influx of Ca^{2+} is an essential step leading to AMPA receptor regulation. Furthermore, more specific Ca^{2+} channel blockers could be used to inhibit AMPA receptor regulation. This would allow the determination of the specific voltage-gated Ca^{2+} channel(s) involved in AMPA receptor regulation by AMPA and v+g.

REFERENCES

Aniksztejn, L., Kleschevnikov, A., Ben-Ari, Y., Represa, A. (1992). Carbachol potentiates NMDA current via protein kinase C in rat hippocampal slices. Soc. Neurosci. Abstr. 18, 256.

Baudry, M. and Lynch, G. (1979). Regulation of glutamate receptors by cations. Nature 279, 748-750.

Bennet, J.P. (1978). Methods in binding studies in neurotransmitter receptor binding. Yamamura, H.I., Enn, S.J., and Kuhar, M.J., eds. New York: Raven Press, 57-90.

Bennett, G.W., Sharp, T., Marsden, C.A. and Parker, T.L. (1983). A manually-operated brain tissue slicer suitable for neurotransmitter release studies. J. Neurosci. Methods 7, 107-115.

Benovic, J.L., Kuhn, H., Weyand, I., Codina, J., Cron, M.G. and Lefkowitz, R.J. (1987). Functional desensitization of the isolated ß-adrenergic receptor by the ß-adrenergic receptor kinase: potential role of an analog for the retinal protein arrestin (48-kDa protein). Proc. Natl. Acad. Sci. USA 84, 8879-8882.

Bird, S.J. and Aghajanian, G.K. (1975). Denervation supersensitivity in the cholinergic septohippocampal pathway: a microiontophoretic study. Brain Res. 100, 355-370.

Bliss, T.V.P. and Collingridge, G.L. (1993). Synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31-39.

Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C. and Heinemann, S. (1990). Molecular cloning and functional expression of glutamate receptor subunit genes. Science 249, 1033-1037.

Boulton, A.A., Baker, G.B., Hrdina, P.D., editors. (1985). General principles of receptor binding. Neuromethods 4. Clifton, NJ: Humanan Press , 2-22.

Bridges, R.J., Stevens D.R., Kahle, J.S., Nunn, P.B., Kadri, M. and Cotman, C.W. (1989). Structure-function studies on N-oxalyl-diamino-dicarboxylic acids and excitatory amino-acid receptors-evidence that Beta-L-ODAP is a selective non-NMDA agonist. J. Neurosci. 9 (6), 2073-2079.

Brorson, J.R., Manzolillo, P.A. and Miller, R. J. (1994). Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. J. Neurosci. 14, 187-197.

Browning, M.D., Bureau, M., Dudek, E.M. and Olsen, R.W. (1990). Protein kinase C and cAMP-dependent protein kinase phosphorylate the ß subunit of the purified γ-aminobutyric acid-A receptor. Proc. Natl. Acad. Sci. USA 87, 1315-1318.

Burt, D. (1980). Basic receptor methods II. Problems of interpretation in binding studies. In: Short course syllabus: receptor binding techniques. Cincinnati, Ohio: Soc. Neurosci. , 53-69.

Bylund, D.B. (1980). Analysis of receptor binding data. In: Short Course Syllabus: Receptor Binding Techniques. Cincinnati, Ohio: Soc. Neurosci., 70-99.

Bylund, D.B. (1992). A practical guide for receptor binding. Biotech Update 5, 188.

Bylund, D.B. and Yamamura, H.I. (1990). Methods for receptor binding. In: Methods in neurotransmitter receptor analysis. New York: Raven Press, 1-35.

Campbell, P.T., Hnatowich, M., O'Dowd, B.F., Caron, M.G., Lefkowitz, R.J. and Hausdorff, W.P. (1991). Mutations of the human ß2-adrenergic receptor that impair coupling to G_S interfere with receptor down-regulation but not sequestration. Molec. Pharmacol. 39, 192-198.

Carpenter, M.B. Core text of Neuroanatomy. (1988). Baltimore: Williams & Wilkins, 390-433.

Catterall, W.A. (1980). Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu. Rev. Pharmacol. Toxicol. 20, 15-43.

Chen, L. and Huang, L.Y. (1992). Protein kinase C reduces Mg^{2+} block of NMDA-receptor channel as a mechanism of modulation. Nature 356, 521-523.

Chetkovich, D.M. and Sweatt, J.D. (1993). NMDA receptor activation increases cyclic AMP in area CA1 of the hippocampus via calcium/calmodulin stimulation of adenylyl cyclase. J. Neurochem. 61(5), 1933-42.

Church, J., Abel-Hamid, E.J. and MacDonald, J.F. (1994). Loperamide blocks high voltage-activated calcium channels and N-methyl-D-aspartate-evoked responses in rat and mouse cultured hippocampal pyramidal neurons. Molec. Pharmacol., 45, 747-757.

Cochet, C., Souvignet, C., Keramidas, M. and Chambaz, E.M. (1986). Altered catalytic properties of protein kinase C in phorbol ester treated cells. Biochem. Biophys. Res. Comm. 134, 1031-1037.

Collingridge, G.L. and Singer, W. (1991). Excitatory amino acid receptors and synaptic plasticity. Trends in Pharmacol. Sci. Special Report , 42-48.

Collins, S., Caron, M.G. and Lefkowitz, R.J. (1991). Regulation of adrenergic receptor responsiveness through modulation of receptor gene expression. Annu. Rev. Physiol. 53, 497-508.

Cox, J.A., Lysko, P.G. and Henneberg, R.C. (1989). Amino acid neurotoxicity at the N-methyl-D-aspartate receptor in cultured neurons: role of voltage-dependent magnesium block. Brain Res. 499, 267-272.

Ebert, B., Madsen, U., Lund, T.M., Lenz, S.M., and Krogsgaard-Larson, P. (1994). Molecular pharmacology of the AMPA agonist, (S)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid [(S)-APPA] and the AMPA antagonist, (R)-APPA. Neurochem. Int. 24, 507-515.

Eccles, J.C. (1976). From electrical to chemical transmission in the central nervous system. The closing address of the Sir Henry Dale Centennial Symposium. Notes Rec. R. Soc. Lond. 30, 219-230.

Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987a). Protein serine/threonine kinases. Annu. Rev. Biochem. 56, 567-613.

Edelman, G.M., Gall, W.E. and Cowan, W.M., eds. (1987b). Synaptic Function. New York: Wiley.

Egebjerg, J., Bettler, B., Hermans-Borgmeyer, I. and Heinemann, S. (1991). Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. Nature 351, 745-748.

Finkbeiner, S. and Stevens, C.F. (1988). Applications of quantitative measurements for assessing glutamate neurotoxicity. Neurobiology 85, 4071-4074.

Forn, J. and Greengard, P. (1978). Depolarizing agents and cyclic nucleotides regulate the phosphorylation of specific neuronal proteins in rat cerebral cortex slices. Proc. Natl. Acad. Sci. USA 75 (10), 5195-5199.

Frandsen, A. and Schousboe, A. (1987). Time and concentration dependency of the toxicity of excitatory amino acids on cerebral neurons in primary culture. Neurochem. Int. 10, 583-591.

Fujisawa, H. (1990). Calmodulin-dependent protein kinase II. BioEssays 12, 27-29.

Fukanaga, K., Soderling, T.R. and Miyamoto, E. (1992). Activation of Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. J. Biol. Chem. 31, 22527-22533.

Gerber, G., Kangrga, I., Ryu, P.D., Larew, J.S.A. and Randic, M. (1989). Multiple effects of phorbol esters in the rat spinal dorsal horn. J. Neurosci. 9, 3606-617.

Gordon, A.S., Davis, C.G. and Diamond, I. (1977). Phosphorylation of membrane proteins at a cholinergic synapse. Proc. Natl. Acad. Sci. USA 74, 263-267.

Greengard, P., Valtorta,F., Czernik, A.J. and Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259, 780-785.

Greengard, P., Jen, J., Nairn, A.C.and Stevens, C.F. (1991). Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. Science 253, 1135-1138.

Guiffrida, R. and Rustoni, A. (1988). Glutamate and aspartate immunoreactivity in corticothalamic neurons of rats. In: Cellular Thalamic Mechanisms. M. Bentivlio and R. Spreafico, eds. Amsterdam: Elsevier, 311-320.

Guiffrida, R. and Rustoni, A. (1989). Glutamate and aspartate immunoreactivity in corticospinal neurons of rat. J. Comp. Neurol. 288, 154-164.

Hablitz, J.J., Tehrani, M.H.J. and Barnes, Jr., E.M. (1989). Chronic exposure of developing cortical neurons to GABA down-regulates GABA/benzodiazepine receptors and GABA-gated chloride currents. Brain Res. 501, 332-338.

Hahn, J.S., Aizenman, E. and Lipton, S.A. (1988). Central mammalian neurons normally resistant to glutamate toxicity are made sensitive by elevated extracellular Ca^{2+}: toxicity is blocked by the N-methyl-D-aspartate antagonist MK-801. Neurobiology 85, 6556-6560.

Hall, R.A., Kessler, M. and Lynch, G. (1992). Evidence that high- and low-affinity DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) binding sites reflect membrane-dependent states of a single receptor. J. Neurochem. 59, 1997-2004.

Hanson, P.I. and Schulman, H. (1992). Neuronal Ca^{2+}/calmodulin-dependent protein kinases. Annu. Rev. Biochem. 61, 559-601.

Hausdorff, W.P., Caron, M.G. and Lefkowitz, R.J. (1990). Turning off the signal: desensitization of ß-adrenergic receptor function. FASEB J. 4, 2881-2889.

Hollmann, M., Hartley, M. and Heinemann, S. (1991). Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. Science 252, 851-853.

Honore, T., Drejer, J., Nielsen, E.O., and Nielsen, M. (1989). Non-NMDA glutamate receptor antagonist ^{3}H-CNQX binds with equal affinity to two agonist states of quisqualate receptors. Biochem. Pharmacol. 38, 3207-3212.

Honore, T., Davies, S.N., Drejer, J., Fletcher, E.J., Jacobsen, P., Lodge, D. and Nielsen, F.E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. Science 241, 701-703.

Honore, T., Lauridsen, J. and Krogsgaard-Larsen, P. (1982). The binding of [^{3}H]AMPA, a structural analogue of glutamic acid, to rat brain membranes. J. Neurochem. 38, 173-178.

Hopfield, J.F., Tank, D.W., Greengard, P. and Huganir, R.L. (1988). Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. Nature 336, 677-680.

Huganir, R.L., Delcour, A.H., Greengard, P. and Hess, G.P. (1986). Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. Nature 321, 774-776.

Huganir, R.L. and Greengard, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. Neuron 5, 55-67.

Hunter, C., Wheaton, K.D. and Wenthold, R.J. (1990). Solubilization and partial purification of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid binding sites from rat brain. J. Neurochem. 54, 118-125.

Hunter, C. and Wenthold, R. (1992). Solubilization and purification of an α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid binding protein from bovine brain. J. Neurosci. 4, 1379-1385.

Ishii, A., Kiuchi, K., Kobayashi, R., Sumi, M., Hidaka, H. and Nagatsu, T. (1991). A selective Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, KN-62, inhibits the enhanced phosphorylation and the activation of tyrosine hydroxylase by 56 mM K$^+$ in rat pheochromocytoma PC12 cells. Biochem. 176, 1051-1056.

Ito, M. and Karachot, L. (1992). Protein kinases and phosphatase inhibitors mediating long-term desensitization of glutamate receptors in cerebellar Purkinje cells. Neurosci. Res. 14, 27-38.

Kachur, J.F., Morgan, D.W., and Gaginella, T.S. (1986). Effects of dextromethorphan on guinea pig ileal contractility *in vitro*: comparison with levomethorphan, loperamide and codeine. J. Pharmacol. Exp. Ther. 239, 661-667.

Kano, M. and Konnerth, A. (1992). Potentiation of GABA-mediated currents by cAMP-dependent protein kinase. Neuroreport 3, 563-566.

Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakmann, B. and Seeburg, P.H. (1990). A family of AMPA-selective glutamate receptors. Science 249, 556-560.

Keler, T. and Smith, C.A.D. (1989). Effects on growth and energy metabolism in untransformed and transformed BALB/c mouse fibroblasts by a novel cytotoxic compound. Cancer Research 49, 7093-7097.

Keller, B.U., Hollmann, M., Heinemann, S. and Konnerth, A. (1992). Calcium influx through subunits GluR1/GluR3 of kainate/AMPA receptor channels is regulated by cAMP dependent protein kinase. EMBO J. 11, 891-896.

Kelso, S.R., Nelson, T.E. and Leonard, J.P. (1992). Protein kinase C-mediated enhancement of NMDA currents by metabotropic glutamate receptors in Xenopus oocytes. J. Physiol. 449, 705-718.

Kennelly, P.J. and Krebs, E.G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. 266, 15555-15558.

Kirkness, E.F., Bovenkerk, C.F., Ueda, T. and Turner, A.J. (1989). Phosphorylation of γ-aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. Biochem. J. 29, 613-616.

Kirkwood, A., Dudek, S.M., Gold, J.T., Aizenman, C.D. and Bear, M.F. (1993). Common forms of synaptic plasticity in the hippocampus and neocortex in vitro. Science 260, 1518-1521.

Kitamura, Y., Miyazaki, A., Yamanaka, Y. and Nomura, Y. (1993). Stimulatory effects of protein kinase C and calmodulin kinase II on N-methyl-D-aspartate receptor/channels in the postsynaptic density of rat brain. J. Neurochem. 61, 100-109.

Klein, W.L., Nathanson, N. and Nirenberg, M. (1979). Muscarinic acetylcholine receptor regulation by accelerated rate of receptor loss. Biochem. & Biophys. Res. Comm. 90, 506-512.

Koh, J-Y., Goldberg, M.P., Hartley, D.M. and Choi, D.W. (1990). Non-NMDA receptor-mediated neurotoxicity in cortical culture. J. Neurosci. 10 (2), 693-705.

Kraft, A.S., Anderson, W.B. Cooper, H.L. and Sando, J.J. (1982). Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. J. Biol. Chem. 257, 13193-13196.

Krieg, W.J.S. (1946). Connections of the cerebral cortex. The albino rat. A topography of the cortical areas. J. Comp. Neurol. 84, 221-275.

Krieger, C., Wagey, R., Lanius, R.A., and Shaw, C.A. (1993). Activation of PKC reverses apparent NMDA receptor reduction in ALS. NeuroReport 4, 931-934.

Krishek, B.J. , Blackstone, C. and Huganir, R.L. (1994). Regulation of GABA$_A$ receptor function by protein kinase C phosphorylation. Neuron 12 (5), 1081-1096.

Lai, C. and Lemke, G. (1991). An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron 6, 691-704.

Lanius, R.A. and Shaw, C. (1992). Characterization and regulation of a high-affinity [^{3}H]-CNQX labeled AMPA receptor in rat neocortex. Molec. Brain Res. 15, 256-262.

Lanius, R.A., Pasqualotto, B.A. and Shaw, C.A. (1993a). Age-dependent expression, phosphorylation and function of neurotransmitter receptors: pharmacological implications. Trends in Pharmacol. Sci. 14(11), 403-408.

Lanius, R.A., Pasqualotto, B.A. and Shaw, C.A. (1993b). A novel mechanism of AMPA receptor regulation: Ionically triggered kinases and phosphatases. NeuroReport 4, 795-798.

Lanius, R.A. and Shaw, C.A. (1993). High-affinity kainate binding sites in living slices of rat neocortex: Characterization and regulation. Neurosci. 55 (1), 139-145.

Leidenheimer, N.J., Browning, M.D. and Harris, R.A. (1991). $GABA_A$ receptor phosphorylation: multiple sites actions and artifacts. Trends Pharmacol. Sci. 12, 84-86.

Liles, W.C. and Nathanson, N.M. (1987). Regulation of muscarinic acetylcholine receptor number in cultured neuronal cells by chronic membrane depolarization. J. Neurosci. 7(8), 2556-2563.

Liman, E.R., Knapp, A.G. and Dowling, J.E. (1989). Enhancement of kainate-gated currents in retinal horizontal cells by cyclic AMP-dependent protein kinase. Brain Res. 481, 399-402.

Linden, D.J. (1994). Long-term synaptic depression in the mammalian brain. Neuron 12, 457-472.

Linden, D.J., Dickinson, M.H., Smeyne, M. and Conner, J.A. (1991). A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron 7, 81-89.

Linden, D.J., Smeyne, M. and Connor, J.A. (1993). Induction of cerebellar long-term depression in culture requires postsynaptic action of sodium ions. Neuron 11, 1093-1100.

Linden, S.J. and Connor, J.A. (1993). Cellular mechanisms of long-term depression in the cerebellum Curr. Opin. Neurobiol. 3, 401-406.

Lipton, S.A. and Rosenberg,P.A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. New Eng. J. of Med. 330, 613-622.

Liron, Z., Wong, E. and Roberts, E. (1988). Studies on uptake of γ-aminobutyric acid by mouse brain particles; toward the development of a model. Brain Res. 444, 119-132.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol Chem. 193, 265-275.

Luqmani, Y.A., Bradford, H.F., Birdsall, N.J. and Hulme, E.C. (1979). Depolarization-induced changes in muscarinic cholinergic receptors in synaptosomes. Nature 277, 481-482.

Lysko, P.G., Cox, J.A., Vigano, A. and Henneberry, R.C. (1989). Excitatory amino acid neurotoxicity at the N-methyl-D-aspartate receptor in cultured neurons: pharmacological characterization. Brain Res. 499, 258-266.

Maloteaux, J.-M. , Octave, J.-N., Gossuin, A., Laterre, C. and Trouet, A. (1987). GABA induces down-regulation of the benzodiazepine-GABA receptor complex in the rat cultured neurons. Eur. J. Pharmacol. 144 , 173-183.

Maren, S., Tocco, G., Standley, S., Baudry, M. and Thompson, R.F. (1993). Postsynaptic factors in the expression of long-term potentiation (LTP): increased glutamate receptor binding following LTP induction in vivo. Proc. Natl. Acad. Sci. USA 90, 9654-9658.

Martin, L.J., Blackstone, C.D., Levey, A.I., Huganir, R.L. and Price, D.L. (1993). AMPA Glutamate receptor subunits are differentially distributed in rat brain. Neurosci. 53, 327-358.

McGlade-McCulloh, E., Yamamoto, H., Tan, S.E. and Brickey, D.A. (1993). Phosphorylation and regulation of glutamate receptors by calcium/calmoldulin-dependent protein kinase II. Nature 362, 640-642.

McIlwain, H., Buchel, L. and Chesire, J.D. (1951). The inorganic phosphate and phosphocreatine of brain especially during metabolism in vitro. Biochem. J. 48, 12-20.

Mehta, A.K. and Ticku, M.K. (1992). Chronic GABA exposure down-regulates GABA-benzodiazepine receptor-ionophore complex in cultured cerebral cortical neurons. Mol. Brain Res. 16, 29-36.

Meldrum, B. and Garthwaite, J. (1991). Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends in Pharmacol. Sci. Special Report , 54-62.

Monaghan, D.T., Bridges, R.J. and Cotman, C.W. (1989). The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 29, 365-402.

Moss, S.J., Smart, T.G., Blackstone, C.D. and Huganir, R.L. (1992). Functional modulation of GABA$_A$ receptors by cAMP-dependent protein phosphorylation. Science 257, 661-665.

Nairn, A.C., Hemmings, Jr., H.C. and Greengard, P. (1985). Protein Kinases in the brain. Ann. Rev. Biochem. 54, 931-76.

Nielsen, E.O., Drejer, J., Cha, J-H J., Young, A.B. and Honore, T. (1990). Autoradiographic characterization and localization of quisqualate binding sites in rat brain using the antagonist [^{3}H]6-7-nitroquinoxaline-2, 3-dione: comparison with (R,S)-[^{3}H]$\propto$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid binding sites. J. Neurochem. 54, 686-694.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661-665.

Olsen, R.W., Szamraj, O. and Houser, C.R. (1987). [^{3}H]AMPA binding to glutamate receptor subpopulations in rat brain. Brain Res. 402, 243-254.

Ortega, A. and Teichberg, V.I. (1990). Phosphorylation of the 49-kDa putative subunit of the chick cerebellar kainate receptor and its regulation by kainatergic ligands. J. Biol. Chem. 265, 21404-21406.

Pasqualotto, B.A., Lanius, R.A. and Shaw, C.A. (1994). Heterologous regulation of cortical AMPA receptors through voltage-gated Ca^{2+} channels. Soc. Neurosci. Abstract, 210.11.

Patneau, D.K., Wright, P.W., Winters, C., Mayer, M.L., and Gallo, V. (1994). Glial cells of the oligodendrocyte lineage express both kainate- and AMPA -preferring subtypes of glutamate receptor. Neuron 12, 357-371.

Petralia, R.S. and Wenthold, R.J. (1992). Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. J. Comp. Neurol. 318, 329-354.

Pelech, S.L., Meier, K.E. and Krebs, E.G. (1986). Rapid microassay for protein kinase C translocation in Swiss 3T3 cells. J. Biol. Chem. 25, 8348-8353.

Pelech, S.L., Samiei, M., Charest, D.L., Howard, S.L. and Salari, H. (1991). Characterization of calcium-independent forms of protein kinase C in phorbol ester-treated rabbit platelets. J. Biol. Chem. 266, 8696-8705.

Porter, N.M., Twyman, R.E., Uhler, M.D. and MacDonald, R.L. (1990). Cyclic AMP-dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons. Neuron 5, 789-796.

Rakic, P. (1988). Specification of cerebral cortical areas. Science 241, 170-176.

Raymond, L.A., Blackstone, C.D. and Huganir, R.L. (1993a). Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. Trends in Neurosci. 16, 147-153.

Raymond, L.A., Blackstone, C.D. and Huganir, R.L. (1993b). Phosphorylation and modulation of recombinant GluR6 glutamate receptors by a cAMP-dependent protein kinases. Nature 361, 637-641.

Reid, K.H., Edmonds, Jr., H.L., Schurr, A., Tseng, M.T. and West, C.A. (1988). Pitfalls in the use of brain slices. Progress in Neurobiology 31, 1-18.

Reymann, K.G., Davies, S.N., Matthies, H., Kase, H. and Collingridge, G.L. (1990). Activation of a K-252b-sensitive protein kinase is necessary for a post-synaptic phase of long-term potentiation in area CA1 of rat hippocampus. European J. Neurosci. 2, 481-486.

Ruiz-Gomez, A., Vaello, M.L., Valdivieso, F. and Mayor, F. (1991). Phosphorylation of the 48k Da subunit of the glycine receptor by protein kinase C. J. Biol. Chem. 266, 559-565.

Saitoh, T. and Changeux, J.P. (1980). Phosphorylation in vitro of membrane fragments of Torpedo mamorata electric organ. Eur. J. Biochem. 105, 51-62.

Scott, J.D. (1991). Cyclic nucleotide-dependent protein kinases. Pharmacol. Therapeutics 50, 123-145.

Seeburg, P.H. (1993). The TiNS/TiPS Lecture: The molecular biology of mammalian glutamate receptor channels. Trends in Neurosci. 16, 359-364.

Shaw, C.A. and Lanius, R.A. (1992). Reversible kinase and phosphatase regulation of brain amino acid receptors in postnatal development. Dev. Brain Res. 70, 153-156.

Shaw, C., Lanius, R.A., Scarth, B.A. and Wilkinson, M. (1992a). The "living" brain slice technique: utility in radioligand receptor characterization and regulation studies. Biotech Update 7, 165-170.

Shaw, C., Pasqualotto, B.A. and Lanius, R.A. (1992b). A role for kinase/phosphatase action in the regulation of brain amino acid receptors. Molec. Neuropharmacol. 2, 297-302.

Shaw, C., Lanius, R.A. and Van den Doel, K. (1994). The origin of synaptic neuroplasticity: crucial molecules or a dynamical cascade? Brain Res. Rev., in press.

Shaw, C. and Scarth, B.A. (1991). Characterization and differential regulation or $GABA_A$ and benzodiazepine receptors in rat neocortex. Mol. Brain Res. 11, 273-282.

Shaw, C.A. and Wilkinson, M. (1994). Receptor characterization and regulation in intact tissue preparations: pharmacological implications. Biochem. Pharmacol., in press.

Shaw, C., Van Huizen, F., Cynader, M.S., Wilkinson, M. (1989). A role for potassium channels in the regulation of cortical muscarinic acetylcholine receptors in an in vitro slice preparation. Mol. Brain Res. 5, 71-83.

Sheardown, M.J., Nielsen, E.O., Hansen, A.J., Jacobsen, P. and Honore, T. (1990). 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline: a neuroprotectant for cerebral ischemia. Science 247, 571-574.

Sieghart, W., Forn, J. and Greengard, P. (1979). Ca^{2+} and cyclic AMP regulate phosphorylation of same two membrane-associated proteins specific to nerve tissue. Proc. Natl. Acad. Sci. 76 (5), 2475-2479.

Siman, R.G. and Card, J.P. (1988). Excitatory amino acid neurotoxicity in the hippocampal slice preparation. Neurosci. 26 (2), 433-447.

Siman, R.G. and Klein, W.L. (1979). Cholinergic activity regulates muscarinic receptors in central nervous cultures. Proc. Natl. Acad. Sci. USA 76, 4141-4145.

Smith, M.K., Colbran, R.J. and Soderling, T.R. (1990). Specificities of autoinhibitory domain peptides for four protein kinases. Implications for intact cell studies of protein kinase function. J. Biol. Chem. 265, 1837-1840.

Soderling, T.R., Tan, S.E., McGlade-McCulloh, E., Yamamoto, H. and Fukanaga, K. (1994). Excitatory interactions between glutamate receptors and protein kinases. J. Neurobiol. 3, 304-311.

Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M.H., Patlak, C.S., Pettigrew, K.D., Sakurdada, O. and Shinohara, M. K. (1977). The [^{14}C]Deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure and normal values in the conscious and anesthetized albino rat. J. Neurochem. 28, 897-916.

Sommer, B., Keinanen, K., Verdoorn, T.A., Wisden, W., Burnashev, N., Herb, A., Kohler, M., Takagi, T., Sakmann, B. and Seeburg, P.H. (1990). Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science 249, 1580-1585.

Song, Y. and Huang, L.-Y. M. (1990). Modulation of glycine receptor chloride channels by cAMP-dependent protein kinase in spinal trigeminal neurons. Nature 384, 242-245.

Staubli, U., Ambros-Ingerson, J., and Lynch, G. (1992). Receptor changes and LTP: an analysis using aniracetam, a drug that reversibly modifies glutamate (AMPA) receptors. Hippocampus 2, 49-58.

Stelzer, A., Kay, A.R. and Wong, R.S.K. (1988). GABA$_A$-receptor function in hippocampal cells is maintained by phosphorylation factors. Science 241, 339-341.

Stephenson, F.A. (1988). Understanding the GABA$_A$ receptor: a chemically gated ion channel. Biochem. J. 249, 21-31.

Stryer, L. (1988). Biochemistry. New York: Freeman.

Sweetnam, P.M., Lloyd, J., Gallombardo, P., Malison, R.T., Gallager, D.W., Tallman, J.F. and Nestler, E.J. (1988). Phosphorylation of the GABA$_A$ benzodiazepine receptor a subunit by a receptor-associated protein kinase. J. Neurochem. 51, 1274-1284.

Swope, S.L., Blackstone, C.D. and Huganir, R.L. (1992). Phosphorylation of ligand gated ion channels: a possible mode of synaptic plasticity. FASEB J. 6, 2514-2523.

Tabuteau, H., de Laneroll, N. and Brines, M. (1993). Depolarization upregulates GABA$_A$ receptor number in living hippocampal slices. Soc. Neurosci. Abstracts 19, 1183.

Tan, Soon-Eng, Wenthold, R.J. and Soderling, T.R. (1994). Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. J. Neurosci. 14 (3), 1123-1129.

Tang, C.-M., Shi, Q.-Y., Katchman, A., and Lynch, G. (1991). Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. Science 254, 288-290.

Tansey, M.G., Word, R.A., Hidaka, H., Singer, H.A., Schworer, C.M., Kamm, K. E.and Stull, J.T. (1992). Phosphorylation of myosin light chain kinase by the multifunctional calmodulin-dependent protein kinase II in smooth muscle cells. J. Biochem. 267, 12511-12516.

Tapley, P.M. and Murray, A.W. (1985). Evidence that treatment of platelets with phorbol ester causes proteolytic activation of Ca^{2+}-activated, phospholipid-dependent protein kinase. Eur. J. Biochem. 151, 419-423.

Tehrani, M.H.J. and Barnes, Jr., E.M. (1988). GABA down-regulates the GABA/benzodiazepine receptor complex in developing cerebral neurons. Neurosci. Lett. 87, 288-292.

Tennant, J.R. (1964). Evaluation of the trypan blue technique for determination of cell viability. Transplantation 2 (6), 685-694.

Teyler, T.J. (1980). Brain slice preparation: hippocampus. Brain Res. Bull. 5, 391-403.

Tocco,G., Maren, S., Shors, T.J., Baudry, M. and Thompson, R.F. (1992). Long-term potentiation is associated with increased [^{3}H]AMPA binding in rat hippocampus. Brain Res. 573, 228-234.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M.and Hidaka, H. (1990). KN-62, 1-[N,O- bis (5-isoquinolinesulfonyl) -N-methyl-L-t yrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. 265, 4315-4320.

Toullec, D., Pianetti, P. Coste, H., Bellevergue, P., Gran-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E. and Loriolle, F. (1991). The bisindolylmaleimide GF 109203x is a potent and selective inhibitor of protein kinse C. J. Biol. Chem. 266, 15771-15781.

Unnerstall, J.R., Niehoff, D.L., Kuhar, M.J. and Palacios, J.M. (1982). Quantitative receptor autoradiography using [^{3}H] labeled ultrafilm: application on multiple benzodiazepine receptors. J. Neurosci. Methods 6 (1-2), 59-73.

Vaello, M-L., Ruiz-Gomez, A. and Mayor, Jr., F. (1992). Glycinergic ligands modulate the rate of phosphorylation of the glycine receptor by protein kinase C. Biochem. Biophys. Res. Comm. 188 (2), 813-819.

Van Haastert, P.J.M., Van Driel, R., Jastorff, B., Baraniak, J., Stec, W.J. and De Wit, R.J. (1984). Competitive cAMP antagonists for cAMP-receptor proteins. J. Biol. Chem. 259, 10020-10024.

Van Huizen, F., Shaw, C., Wilkinson, M. and Cynader, M. (1989). Characterization of muscarinic acetylcholine receptors in rat cerebral cortex with concomitant morphological and physiological assessment of tissue viability. Mol. Brain Res. 5, 59-69.

Van Huizen, F., Wilkinson, M., Cynader, M. and Shaw, C. (1988). Sodium channel toxins veratrine and veratridine modify opioid and muscarinic but not ß-adrenergic binding sites in brain slices. Brain Res. Bull. 21, 129-132.

Walaas, S.I., Nairn, A.C. and Greengard, P. (1983a). DARP-32, a dopamine- and adenosine 3',5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain region. I. regional and cellular distribution in the rat brain. J. Neurosci. 3, 302-311.

Walaas, S.I., Nairn, A.C. and Greengard, P. (1983b). Regional distribution of calcium- and cyclic adenosine 3', 5' -monophosphate-regulated protein phosphorylation systems in mammalian brain. J. Neurosci. 3, 291-301.

Walton, K. and Fulton, B. (1983). Hydrogen peroxide as a source of molecular oxygen for in vitro mammalian CNS preparations. Brain Res. 278, 387-393.

Wang, L.Y., Salter, M.W. and MacDonald, J.F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. Science 253, 1132-1138.

Wang, Y., Qiang, G., Fei., M., Haugland, R. P. and Cynader, M.S. (1994). Activity-dependent expression and distribution of M1, muscarinic ACh receptors in visual cortex neuronal cultures. J. Neurosci. 14 (7), 4147-4158.

Wenthold, R.J., Yokotani, N., Doi, K. and Wada, P. (1992). Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies. Evidence for a hetero-oligomeric structure in rat brain. J. Biol. Chem. 267, 501-507.

Wiesel, T.N. and Hubel, D.H. (1963). Single-cell responses in striate cortex of kittens deprived of vision in one eye. J. Neurophysiol. 26, 1003-1017.

Wilkinson, M., Jacobson, W. and Watson-Wright, W. (1986). Minireview: tissue slices in radioligand binding assays: studies in brain, pineal, and muscle. Life Sci. 39, 2037-2048.

Wurtman, R.J. and Fernstrom, J.D. (1976). Control of brain neurotransmitter syntheses by precursor availability and nutritional state. Biochem. Pharmacol. 25, 1691-1696.

Yamamura, H.I., Enna, S.J. and Kuhar M.J. (1990). Methods in neurotransmitter receptor analysis. New York: Raven Press.

Yang, J., Logsdon, C.D., Johansen, T.E. and Williams, J.A. (1994). Human m3 muscarinic acetylcholine receptor carboxyl-terminal threonine residues are required for agonist-induced receptor down-regulation. Molec. Pharmacol. 44, 1158-1164.

Young, A.B. and Fagg, G.E. (1991). Excitatory amino acid receptors in the brain: membrane binding and receptor autoradiographic approaches. Trends in Pharmacol. Sci. Special Report, 18-24.

Young, S. and Kuhar, M.J. (1987). A new method for receptor autoradiography: [^{3}H] -opioid receptors in rat brain. Brain Res. 179, 255-270.

Zaczek, R., Arlis, S., Markl, A., Murphy, T., Drucker, H. and Coyle, J.T. (1987). Characteristics of chloride-dependent incorporation of glutamate into brain membranes argue against a receptor binding site. Neuropharmacol. 26, 281-287.

Zbinden, G., ed. (1992). The brain in bits and pieces. In vitro techniques in neurobiology, neuropharmacology and neurotoxicology. Zollikon, Switzerland: MCT Verlag., 13-15