Assessment of the structure and function of kainate-type glutamate receptors GluR6

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Abstract

Glutamate is the main excitatory neurotransmitter in the brain and is essential in most physiological processes. Recent evidence indicates that the structure of ionotropic glutamate receptors (GluRs) is unique among the ligand-gated ion channel superfamily. The C-terminus of GluR is intra- rather than extracellular and the pore forming segment (M2) forms a hairpin structure in the membrane. In addition, several studies indicate that the M3-M4 loop (the segment connecting transmembrane 3 and 4) is extracellular. However, other studies suggest that residues within the M3-M4 loop are sites of phosphorylation and modulation of channel function by intracellular protein kinases, and therefore the loop is intracellular. To reconcile these conflicting data, we hypothesized that GluR may have a dynamic structure in which, upon agonist binding to and activation of the channel, a portion of the M3-M4 loop containing putative phosphorylation sites translocates across the membrane to the cytosolic side. In order to test this hypothesis, we used site directed mutagenesis to mutate a putative PKA phosphorylation site located in M3-M4 loop of GluR6 (Ser684) to Cys, allowing further modification of this residue by Cys-specific reagents. Using a combined biochemical and electrophysiological approach, we determined that in the ligand-unbound state of GluR6(S684C), Cys-684 is accessible from the extracellular side to modification by a Cys-specific biotinylating reagent as well as by streptavidin. In addition, we found that this residue becomes inaccessible for biotinylation upon agonist binding and activation of the channel. However, our electrophysiological data indicate that translocation of C684 to the cytoplasmic side is unlikely. The results of our biochemical and electrophysiological experiments indicate
that residue 684 in the M3-M4 loop is located extracellularly in the channel’s ligand unbound, closed state, as well as ligand-bound, activated or desensitized state.

In addition, we analyzed the role of cysteine residues in the normal function of GluR6 receptors. Cys-residues have been shown to be important in the function of several ligand-gated ion channels. Our data indicate that cysteine residues within the M3-M4 loop and N-terminal region contribute to the function and pharmacological properties of GluR6 receptors. Mutation of GluR6 Cys-719 to serine or threonine (C719S, C719T) resulted in a change in the EC50 for glutamate and slowed desensitization of the channel. Other mutations (C773S, C316S and C719S/C773S) resulted in loss of protein expression. Moreover, our data suggest that Cys-719 and Cys-65 form disulfide bonds with other cysteine residues in the M3-M4 loop and N-terminal part of the receptor. These results increase our understanding of the structure and function of glutamate receptors and may prove useful in development of drugs specific for glutamate receptor subunits.
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CHAPTER 1

Introduction

Signal transduction at neuronal synapses involves release of chemical neurotransmitter from the pre-synaptic cells that bind to protein receptors on the post-synaptic cell causing electrochemical changes in the post-synaptic cells. Involvement of acidic amino acids in modulation of neuronal activity was first described by Hayashi (1954) who showed a convulsant effect of glutamate on cerebral cortex of monkeys. He infused glutamate to certain area of the cerebral cortex in monkeys and observed limb movement somatotopically related to the area of infusion. However, a role for L-glutamate as neurotransmitter was thought to be unlikely due to similar action of L- and D-isomers and the fact that L-glutamate is involved in cell metabolism. It was thought that the effects of amino acids on neurons were non-specific since a high concentration of the amino acids was needed to activate neurons. However, the levels of L-glutamate and L-aspartate were found to be high in dorsal root afferent neurons and spinal interneurons, inconsistent with a purely metabolic role for these amino acids (Curtis and Johnson, 1974).

Further neurochemical and electrophysiological studies confirmed the role of glutamate as a neurotransmitter in synaptic transmission. Ionophoretic studies by Curtis et al. (1959) showed excitatory action of glutamate on single neurons in the central nervous system. They used microelectrophoretic techniques to deliver L-glutamate and L-aspartate from the tips of a multibarrel electrode to the extracellular region of single neurons in the brain and recorded the
action potential firing of the single neuron. Their experiments demonstrated a direct excitatory action of glutamate on the postsynaptic neurons.

Subsequent experiments with glutamate analogues such as quisqualate, kainate and N-methyl-D-aspartate (NMDA) elucidated the structural requirements for receptor activation. These requirements include a diacidic amino acid with \( \alpha \)-amino and \( \alpha \)-carboxyl groups and a second acidic group in the \( \beta \) or \( \gamma \) position. The second acidic group could be carboxyl, sulfonate, or sulfinate (McLennan, 1983; Curtis and Watkins, 1960). Thus, dicarboxylic amino acids such as glutamate and aspartate would be excitatory and amino acids such as \( \gamma \)-amino butyric acid (GABA) in which the \( \alpha \)-carboxyl group is removed would be inhibitory. These experiments also showed different potencies of the glutamate analogues on subsets of neurons indicating a role for the amino acids as neurotransmitter. Later, transporter systems for L-glutamate and L-aspartate were described (Logan and Snyder, 1971). These transporter systems affect the kinetic properties of responses to exogenously applied L-glutamate by actively reducing synaptic levels of the amino acids. Further evidence to support a role for glutamate in neurotransmission comes from ligand binding studies using \([^{14}\text{C}]\) L-glutamate and \([^{3}\text{H}]\) L-glutamate and demonstrating the presence of high affinity glutamate binding sites on neuronal membranes (Michaelis et al. 1974).

Crucial evidence for the role of L-glutamate in synaptic transmission comes from the effect of glutamate receptor antagonists. The development of antagonists led to the description of several subtypes of glutamate receptors and their role in normal functioning of the brain and in disease. Glutamate receptors mediate the majority of excitatory synaptic transmission in mammalian central nervous system (CNS). These receptors are essential in most physiological
processes such as sensory perception, learning and memory, and movement (Mayer and Westbrook, 1987; Monaghan et al., 1989; Olney 1990; Choi 1992; Nakanishi 1992).

Recent evidence has elucidated the role of specific glutamate receptor subtypes in several physiological processes. Li et al. (1999) showed that glutamate receptors mediate sensory synaptic transmission from spinal cord to the brain. DeVries and Schwartz (1999) have shown that cone receptors in mammalian retina use glutamate as a neurotransmitter to transmit signals to bipolar cells. Several other studies have described glutamate neurotransmission in hippocampal regions, cerebellum, thalamic areas and neocortex (Clarke et al. 1997; Vinges and Collingridge 1997; Castillo et al. 1997).

Glutamate neurotransmission has been shown to contribute to neuronal plasticity and neurotoxicity. Long term potentiation (LTP) and long term depression (LTD) in the hippocampus, as well as in the cerebellum, exemplify glutamate-induced neuronal plasticity. LTP and LTD are thought to underlie the processes of learning and information storage in the brain (Bliss and Collingridge 1993; Nakanishi 1992). Role of glutamate receptors in developmental plasticity has been studied using development of stem cells as well as visual systems (Kandel and O’Dell 1992). Sensory pathways in the brain generally develop from refinement of synaptic connections through activity-dependent changes in developing neuronal groups. Ca$^{2+}$ influx through a subtype of glutamate receptors, the NMDA receptors, has been shown to activate cellular processes that result in refinement of synaptic connections. It has been shown that eye-specific segregation of retinal ganglion cells is dependent on NMDA receptors and that blockade of NMDA receptors results in desegregation of eye-specific stripes (Cline et al. 1987). In addition, application of NMDA receptors antagonists has been shown to disrupt experience-dependent plasticity of kitten striate cortex (Kleinschmidt et al. 1987).
Several studies have used transgenic mice to examine the role of glutamate receptors in neuronal plasticity and development. Ablation of genes encoding a subtype of glutamate receptors, NMDAR1, has been shown to disrupt normal neuronal development in mice (Li et al. 1994). Moreover, synaptic activity mediated by Ca\(^{2+}\) permeable glutamate receptors has been shown to be essential for the formation of neuronal pattern in the mammalian brain. Mice defective in a Ca\(^{2+}\) permeable glutamate receptor subtype, NMDA receptor ε2 subunit, showed impairment of suckling response and died shortly after birth. In addition, formation of normal pattern of trigeminal neurons as well as hippocampal LTD were disrupted in these animals (Kutsuwada et al. 1996).

It is well known that excess glutamate can be neurotoxic and that excessive activation of glutamate receptors can trigger neuronal degeneration and cell death. In a variety of acute and chronic disorders such as ischemic stroke, epilepsy, hypoglycemia, and trauma, high concentration of extracellular glutamate results in extensive stimulation of glutamate receptors and degeneration of neurons (Choi 1994). The molecular mechanisms underlying neuronal degeneration have been studied using tissue culture and brain slice preparations. A number of different pathways are associated with glutamate neurotoxicity and several factors may determine the vulnerability of selective neuronal populations to excitotoxic insults. Some of these factors include glutamate receptor subtypes, buffering mechanisms for intracellular Ca\(^{2+}\), and metabolic processes leading to generation of free radicals as well as free radical scavengers (Coyle and Puttfarcken 1993).

Glutamate-induced neuronal death has been observed to occur in an acute and a time delayed form. These two forms differ in the time course and ionic dependence of neurotoxicity.
In the acute form, over-activation of glutamate receptors results in large influx of $\text{Na}^+$ and $\text{Cl}^-$ followed by massive influx of water resulting in the swelling and osmotic lysis of the cell (Rothman et al. 1987). In the delayed form of neuronal degeneration, a gradual cell death over several hours occurs following brief exposure to a high glutamate concentration or prolonged exposure to a low concentration of glutamate (Choi et al. 1987). Excessive $\text{Ca}^{2+}$ influx has been implicated as a cause of neuronal injury in the delayed form of neuronal degeneration. $\text{Ca}^{2+}$ influx can be mediated by glutamate receptor subtypes, NMDA and AMPA/kainate receptors, as well as by voltage-gated $\text{Ca}^{2+}$ channels. Although the rise in intracellular $\text{Ca}^{2+}$ need not be prolonged to induce neuronal death (Michaels and Rothman, 1990), the rate of $\text{Ca}^{2+}$ influx, as well as intracellular $\text{Ca}^{2+}$ levels, has been shown to underlie induction of injury in cortical neurons. Recent observations with low-affinity fluorescent $\text{Ca}^{2+}$ indicators indicates that neurotoxic effect of glutamate receptor activation is associated with elevation of intracellular $\text{Ca}^{2+}$ to micromolar concentrations (Hyrc et al. 1997). $\text{Ca}^{2+}$ dependent delayed neuronal death has been shown to involve a number of different pathways. These include activation of transcription factors specific for a cell death program (Schreiber and Baudry, 1995), generation of free radicals, and activation of degradative enzymes such as proteases, phospholipases, and endonucleases (Coyle and Puttfarken 1993).

Several studies have linked activation of glutamate receptors to a number of processes leading to oxidative stress. Oxidative stress has been implicated as a causal factor in neuronal degeneration. The rise in intracellular $\text{Ca}^{2+}$ levels upon glutamate receptor activation can activate phospholipase A2 (PLA2) resulting in the release of arachidonic acid and subsequent production of free radicals. In addition, elevated $\text{Ca}^{2+}$ levels can activate proteases such as calpain which
converts xanthine dehydrogenase to xanthine oxidase leading to release of the oxygen radical, \( \text{O}_2^- \) (Dumuis et al. 1988).

A potential source of free radical production is the mitochondrial membrane. Mitochondria are involved in the concerted consumption of oxygen and production of energy in the form of ATP as well as mobilization of \( \text{Ca}^{2+} \). Activation of glutamate receptors and depolarization of the cell increases ATP consumption which may lead to depletion of mitochondrial ATP, increasing oxidative phosphorylation and subsequent oxygen radical production. Moreover, since impairment of energy metabolism increases generation of free radicals (Coyle and Puttfacken 1993), dysfunction of mitochondria may be a main event in glutamate neurotoxicity. It has been suggested that glutamate-induced elevation of intracellular \( \text{Ca}^{2+} \) levels may cause mitochondrial \( \text{Ca}^{2+} \) overload leading to the production of injurious oxygen radicals (Carriedo et al. 1998). Moreover, glutamate-induced \( \text{Ca}^{2+} \) overload as well as depolarization of mitochondrial membrane potential has been shown to closely parallel neuronal death (Schinder et al. 1996).

Selective blockade of the glutamate receptor subtype, NMDA receptors, has been shown to reduce glutamate-induced neurotoxicity (Michaels and Rothman, 1990). Moreover, glutamate receptor antagonists have been shown to have a neuroprotective effect in animal models of brain ischemia, epilepsy, and Parkinson disease. Glutamate neurotoxicity may underlie slowly progressive neurodegenerative disorders such as Alzheimer's disease and Huntington's disease (Dingledine et al. 1999). Therefore, glutamate receptors play important roles in the normal function and dysfunction of the brain.
1.1. Glutamate receptors: physiology, pharmacology and molecular biology.

The GluRs are divided into two major classes: (1) G-protein coupled metabotropic (mGluRs) receptors which trigger second messenger pathways leading to the production of inositol triphosphate (IP3) or regulation of cAMP levels; (2) ion channel forming ionotropic (iGluRs) receptors. The ionotropic glutamate receptors (iGluRs) were first classified as NMDA and non-NMDA receptors. Molecular cloning and development of drugs that could differentiate non-NMDA receptors resulted in classification of these receptors into different subtypes. Based on their pharmacological and physiological properties, the iGluRs have been grouped into three subclasses: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate (KA) receptors. These receptors are formed by heteromultimeric complexes of homologous subunits: NR1 with NR2A-D for NMDA; GluR1-4 for AMPA; GluR5-7 and KA-1, KA-2 for kainate (Nakanishi et al. 1990; Seeburg 1993; Hollmann and Heinemann 1994; Bennett and DINGledine 1995; Sutcliff et al. 1996).

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1.2. The NMDA receptors.

The NMDA receptors can be generated in vitro as heteromultimeric structures from two subunit types, NR1 and one or more of the four NR2 subunits. The common NR1 subunit occurs in eight different splice variants that have different pharmacological properties (Hollmann et al. 1993; Dingledine et al. 1999). These receptors display a number of special characteristics that are important in normal functioning of the nervous system. These characteristics include voltage-dependent responses to agonist due to the sensitivity to extracellular Mg\(^{2+}\) which blocks the channel in a voltage-dependent manner (Mayer and Wesbrook 1987); requirement for both agonist (glutamate or NMDA) and glycine as co-agonist to activate the channels (Johnson and Ascher 1987); and high Ca\(^{2+}\) permeability. The increase in the intracellular Ca\(^{2+}\) upon activation of NMDA receptors initiates a variety of intracellular processes responsible for plasticity as well as excitotoxic cell death (Choi 1994).

1.3. The AMPA receptors.

The AMPA receptors can be generated as homomeric or heteromeric structures in vitro by expressing one subunit or by co-expressing any two of the four subunits, respectively (Keinanen et al. 1990). These receptors desensitize rapidly and profoundly in response to agonist. An alternatively spliced exonic sequence of ~38 amino acids results in two major versions of these subunits named flip and flop. The flip form predominates before birth and the expression of this form is continued throughout life. The flop form is expressed from early postnatal stages
reaching similar levels as the flip form in adult animals. The flip forms of most subunits show slower desensitization kinetics than the flop forms (Sommer et al. 1990). In addition, RNA editing plays an important role in the diversity and function of the AMPA receptor subunits. In GluR2 subunits, a glutamine residue in the pore region of the channel is edited to arginine (Q/R site) resulting in Ca\textsuperscript{2+} impermeability and a linear current-voltage (I-V) relationship (Seeburg 1993). Increased Ca\textsuperscript{2+} permeation through AMPA receptors lacking the GluR2 subunit may underlie a number of pathological conditions including kindled seizures and neurotoxic cell death (Pellegrini-Giampietro et al. 1997).

1.4. The Kainate receptors (KAR).

Early pharmacological studies led to the classification of iGluRs as NMDA and non-NMDA receptors. Advances in molecular biology and cloning of several different iGluRs and development of drugs that could differentially activate non-NMDA receptors resulted in classification of these receptors as AMPA and Kainate receptors. Due to lack of antagonists that could distinguish these two receptor subtypes, the role of KAR in synaptic transmission was not well understood. However, with recent advances, including development of GluR6 gene knockout mice, as well as drugs that specifically block or activate AMPA or kainite receptors, the physiological role of the KARs is becoming more clear.

In a recent study, Bureau et al. (1999) have used GluR6-deficient mice to examine kainate receptor-mediated responses in hippocampus. These workers compared the action of kainate receptor agonists, kainate and domoate, on hippocampal slices of wild-type and GluR6
deficient mice and demonstrated the presence of functional GluR6 receptors and their involvement in synaptic transmission in the hippocampus. In addition, they showed that kainate receptor activation increases the frequency of spontaneous inhibitory postsynaptic currents (IPSCs) in the CA1 field of both wild-type and GluR6-deficient mice. Their results indicate the presence of functional GluR5 receptors in the inhibitory interneurons of certain hippocampal regions. In addition, their study indicates that distinct kainate receptor subtypes may mediate functionally antagonistic effects in the hippocampal CA1 region.

In another study, Mulle et al. (1998) have used GluR6-deficient mice to study the role of kainate receptors in the synaptic physiology of hippocampus and in excitotoxic processes. They compared susceptibility of wild-type and GluR6-deficient mice to systemic administration of kainate. Administration of kainate to rodents induces a well-characterized seizure. These workers compared onset of seizures as well as activation of immediate early genes (IEGs) in hippocampal regions of wild-type and GluR6-deficient mice and showed that GluR6-deficient mice are less susceptible to systemic infusion of kainate. Their study demonstrates the importance of the GluR6 subunit in epileptogenic effects of kainate.

Several other studies have also shown the physiological role of KARs in the hippocampus where repetitive activation of mossy fibers produces kainate receptor-mediated excitatory postsynaptic currents (EPSCs; Castillo et al. 1997; Vinges and Collingridge, 1997). The role of kainate receptors in mediation of synaptic transmission in mammalian retina has been investigated by DeVries and Schwartz (1999). These workers have made electrophysiological recordings from cone cells and synaptically connected bipolar cells in slices of retina from squirrel. In this study, a selective inhibitor of NMDA receptors, 2-amino-5-phosphonovaleric acid (AP5), had no effect on the synaptic transmission. As well, the postsynaptic current/voltage...
relationship was linear but not rectifying indicating that the current was mediated by AMPA/kainate receptors. Moreover, in order to make a distinction between AMPA- and kainate-mediated responses, they used a selective non-competitive blocker of AMPA receptors as well as cyclothiazide, a modulator of AMPA receptors. Their data indicated that synaptic transmission between cone and bipolar cells was mediated by kainate receptors.

Mediation of sensory synaptic transmission in mammalian spinal cord has also been shown to involve kainate receptors. Li et al. (1999) have made patch clamp recordings from neurons in the superficial dorsal horn of the spinal cord. These neurons receive afferent input from the primary sensory neurons in the periphery. In this study, the spinal slices were obtained from rats at different postnatal stages. To show kainate receptor-mediated responses, they superfused the slices with a selective blocker of the NMDA receptors (AP5), and two selective non-competitive antagonists of AMPA receptors, GYKI53655 and SYM2206. They applied a high-intensity single shock stimulation to primary afferent sensory fibers and recorded EPSCs from the superficial dorsal horn of the spinal cord. Their results indicate that kainate receptors are involved in the transmission of noxious stimuli from the periphery to the brain.

Homomeric KAR can be reconstituted in vitro by expressing GluR5, GluR6, or GluR7 subunits. In vitro, expression of KA1 or KA2 does not generate functional channels. However, when co-expressed with one of the GluR5, GluR6, or GluR7 subunits, heteromeric channels can be generated. Alternative splicing and RNA editing of KAR subunits adds to the structural and functional diversity of glutamate receptors. At least nine different C-terminal splice variants are found among the KAR-subunits GluR5-7 (Dingledine et al. 1999). RNA editing is observed for GluR5 and GluR6 receptors in the TM2 domain (Q/R site). GluR6 receptors contain two additional sites in the TM1 domain that are modified by RNA editing. An isoleucine is changed
to valine (I to V) and a tyrosine to cysteine (Y to C). As the result of RNA editing eight different forms of GluR6 exist, one genomically encoded and seven RNA-edited forms. Although the unedited kainate receptors (I, Y, Q) are present in both embryonic and adult brain, most of the kainate receptors in the adult CNS are edited in at least one position. In the adult brain, 50-60% of GluR5 and 70-90% of GluR6 are found in the edited forms. The fully edited form of GluR6 (V, C, R) accounts for ~65% of all GluR6 receptors in the rat brain while other edited forms constitute ~25% (Chittajalla et al. 1999). The RNA editing (Q/R site) has interesting functional consequences in both GluR5 and GluR6 receptors. Homomeric receptors containing edited subunits, GluR5 (R) or GluR6 (R), display low $Ca^{2+}$ permeability, a linear rather than inwardly rectifying current, and increased permeability to $Cl^-$ (Kohler et al. 1993; Chittajalla et al. 1999).

Similar to AMPA receptors, homomeric KARs desensitize rapidly and almost completely in response to agonists (Heckmann et al. 1996). Unlike homomeric KARs, heteromeric KARs generated by GluR6 and KA2 subunits in vitro are activated by AMPA indicating that subunit composition in vivo may affect pharmacological properties of these receptors (Seeburg 1993).

**1.5. Subunit distribution of glutamate receptors:**

**1.5.1 The NMDA receptor subunit localization.**

Although the exact subunit composition of native iGluRs is still under investigation, in situ hybridization and immunostaining have revealed relative distributions of the subunits. The NR1 mRNA is expressed at high levels in most neuronal cells (Moriyoshi et al. 1991; Petralia et al. 1994). The NR2 mRNAs, however, show spatial and temporal distribution in the developing
and adult brain as well as in the spinal cord (Monyer et al. 1992; Sugihara et al. 1992; Monyer et al. 1994). The mRNA for the NR2A subunit is expressed predominantly in hippocampus and cerebellum while the NR2B mRNA is expressed highly in thalamic nuclei (Moriyoshi et al. 1991). The NR2C mRNA is expressed largely in granule cells of cerebellum and the NR2D mRNA is expressed mainly in lower brain stem regions and the spinal cord (Tolle et al. 1993). The NR2 subunit expression is also developmentally regulated. Prenatally, NR2B and NR2D mRNAs are highly expressed but the expression level decreases in adults. The NR2A and NR2C mRNAs are expressed postnatally and expression increases until adulthood (Monyer et al. 1994; Zhong et al. 1995).

1.5.2. The AMPA receptor subunit localization.

The AMPA receptor subunits are widely but differentially expressed during development and in the adult CNS. GluR2 and GluR4 mRNAs are abundant in the neocortex and cerebellar granule cells whereas GluR1 and GluR3 mRNA are expressed at lower levels. In the hippocampus, the CA3 region and dentate gyrus express high levels of GluR1-3 but not GluR4, whereas the CA1 region expresses high levels of GluR4 mRNA. The pattern of mRNA for AMPA receptor subunit distribution in the brain approximates the $^3$[H] AMPA binding sites. During development, GluR1-4 genes are expressed by embryonic day 10 (E10) in both the central and peripheral nervous system (Bettler et al. 1990). Postnatally there is a general increase in expression of all four subunit mRNAs. However, there is a developmental increase in the amount of the Ca$^{2+}$ impermeable subunit, GluR2, from prenatal low levels to high levels in the
adult brain. Ca\(^{2+}\) influx through embryonic AMPA receptors may play a role in the development of synaptic connections in the fetal brain (Hollmann and Heinemann 1994).

1.5.3. The Kainate receptor subunit localization.

Kainate receptor subunits are differentially expressed throughout the brain. In situ hybridization experiments have described the relative distribution of five KAR-subunit genes in the developing and adult brain. Specific oligonucleotide probes have been used to examine regional patterns of high affinity KAR subunit mRNA in a series of coronal sections of rat brain (Wisden and Seeburg, 1993). Comparison of the distribution of KAR mRNA indicated that these receptors are expressed in almost all central neurons. However, the most prominent regions expressing KAR subunits are the caudate and putamen, nuclei of basal ganglia, and hippocampus. The medium spiny neurons of the caudate and putamen express high levels of GluR6 mRNA. GluR5 mRNA are found in cingulate and piriform gyrus, the subiculum, lateral septal nuclei, suprachiasmatic nuclei, the tegmental nuclei, and hippocampal stratum oriens and stratum radiatum. GluR6 mRNA is also widely expressed in the hippocampal pyramidal cell layer, dentate gyrus, and in the cerebellar granule cells. GluR7 gene occurs mainly in the inner cortical layer, subiculum, caudate putamen, reticular thalamus, pontine nuclei, and some cells in the cerebellum. KA1 mRNA is expressed mainly in the hippocampal CA1 field and dentate gyrus. Inner cortical layers and cerebellar Purkinje cells also express KA1 mRNA. KA2 mRNA is highly expressed in layers II-IV of neocortex, dentate granule cells, CA1 and CA3 fields of
hippocampus, suprachiasmatic and hypothalamic nuclei, locus coeruleus, dorsal raphe, and granule cells of cerebellum.

Two other groups have performed in situ hybridization experiments with specific antisense RNA-probes to determine the pattern of mRNA expression for five KAR subunits in hippocampal regions of mice (Bureau et al., 1999; Mulle et al. 1998). Their studies indicate that the expression level of GluR6 mRNA in the CA3 field is among the highest levels in the brain. GluR6 is also highly expressed in the CA1 field and in dentate granule cells. GluR5 mRNA is highly expressed in most hippocampal interneurons in the stratum oriens and stratum radiatum. KA2 mRNA is most abundant in CA1 and CA3 regions as well as in dentate gyrus, whereas GluR7 is expressed mainly in dentate gyrus. KA1 mRNA is also highly expressed in the CA3 regions with lower levels in CA1 and dentate gyrus.

Moreover, radiolabeled ligand binding and immunostaining have been used to described the pattern of protein expression for kainate receptor subunits. Bahn et al. (1994) have examined and compared the pattern of KAR mRNA and protein expression in the developing and adult rat brain using antisense oligonucleotides for each of the five high affinity KAR subunits and $^3$[H] kainate autoradiography. The pattern of the five KAR subunit mRNAs largely matches the autoradiographic pattern of $^3$[H] Kainate sites in the rat brain. They have shown that KAR subunits are highly expressed in layer I and inner laminae of the neocortex, cingulate cortex, reticular thalamus, and cerebellar granule cell layer. The most prominent regions expressing the KAR subunit, GluR6, are caudate/putamen and hippocampus.

Immunohistochemical experiments using anti-GluR5, anti-GluR6/7 and anti-KA2 antibodies have localized KAR subunits in the neocortex and at the postsynaptic membrane and
dendritic spines in CA1 and CA3 fields of hippocampus. Siegel et al. (1995) have described the pattern of immunoreactivity for GluR5-7 subunits in the monkey hippocampus using an antibody raised against the N-terminal domain of GluR5 receptors. The antibody could recognize GluR5-7 receptors. Their study indicated that the somatodendritic compartment of dentate granule cells express GluR5-7 receptors. Non-pyramidal cells in the stratum oriens, stratum radiatum, and neuroglial cells in the alveolus and fimbria were labeled with the antibody. Intense immunoreactivity, however, was obtained in the pyramidal cells of CA1-CA3 fields and in the subiculum.

In another study, Huntley et al. (1993) have used anti-GluR5/6/7 antibody to examine immunocytochemically, at the light and electron microscopic levels, the regional and cellular distribution of KAR subunits in monkey neocortex. This study indicated distinct and specific cellular, laminar, and regional immunoreactivity of KAR subunits. Immunostaining of KAR subunits was observed in the pyramidal cell body and apical dendrites, with intense staining of postsynaptic dendrites concentrated in layers II, III, and V. Other neocortical layers, I, IV, and VI, were stained least intensely. In addition, motor areas and higher order association areas of the frontal, parietal, and occipital cortex were labeled more intensely than the somatosensory areas and visual cortex.

1.6. The structure of ionotropic glutamate receptors.

Recent evidence indicates that the transmembrane topology of iGluR is unique among the members of ligand-gated ion channel superfamily. Early structural studies have used
hydrophobicity plot analysis to gain insight into the possible structure of iGluRs. Due to lack of experimental data, the nicotinic acetylcholine receptor has been used as a model to assign iGluR topology. Accordingly, a four transmembrane (4TM) model with N- and C-termini located extracellularly had been proposed (Hollmann et al. 1990). Several lines of evidence, however, contradicted the 4TM topology proposed earlier. Antibodies raised against N- and C-termini of GluRs have been used during immunocytochemical analysis to localize iGluRs. Staining with C-terminal domain antibodies indicated that this domain is intracellular (Petralia and Wenthold, 1992). Molnar et al. (1994) have used N-and C-termini anti-GluR1 antibodies in their immunocytochemical study of brain tissue sections at the electron microscopy level. Their study indicated that immunoreactivity of C-terminal anti-GluR1 was restricted to intracellular sites, while the N-terminal anti-GluR1 labeled the extracellular face of the synaptic cleft. Moreover, C-terminal anti-GluR1 and anti-GluR2 antibodies did not label cultured hippocampal neurons unless the cells were permeabilized (Craig et al. 1993). Taken together, these data indicated that while the N-terminus is extracellular, the C-terminus in located intracellularly (Figure 1).

Further phosphorylation studies have confirmed an intracellular location for the C-terminus. Tingley et al. (1993) have examined protein kinase C (PKC) phosphorylation of NMDA receptor subunit, NR1, in cortical neurons and transiently transfected HEK-293 cells. They have shown that an alternatively spliced exon in the C-terminal domain regulates phosphorylation of the receptor by PKC, indicating an intracellular location for the C-terminus. In addition, Roche et al. (1996) have characterized multiple phosphorylation sites of the GluR1 C-terminal domain by PKC and by cAMP-dependent protein kinase (PKA). To account for the PKC phosphorylation of the C-terminal domain, a topological model in which the C-terminus is intracellular and the TM4 does not cross the membrane was proposed (Tingley et al. 1993).
model places the segment connecting TM3 to TM4 (M3-M4 loop) intracellular. However, further topological studies discussed below indicated that at least part of the M3-M4 loop is located extracellularly.

The original assumption of homology between nAchR and iGluR led to the idea that the TM2 domain lines the pore region. Several studies have confirmed the TM2-domain as the pore region of the channel. Hume et al. (1991) have identified sites in the TM2 domain of AMPA receptors that control Ca\(^{2+}\) permeability of the channels. They have shown that the presence of an arginine (R) or a glutamine (Q) residue in TM2 alters Ca\(^{2+}\) permeability of the receptors. In another study, Burnashev et al. (1992) have shown that Ca\(^{2+}\) permeability and voltage-dependent Mg\(^{2+}\) block of NMDA receptors were mediated by an asparagine (N) residue located within TM2 at a position homologous to the AMPA receptor R/Q site.

More data for the TM2 domain comes from studies by Bennett and Dingledine (1995) who have used a combination of N-glycosylation and epitope tagging to examine the topology of the AMPA receptor subunit, GluR3. They truncated GluR3 at both ends of each of the putative transmembrane domains and fused a non-topogenic domain of prolactin to form the C-terminus of the truncated protein. The fusion proteins were then translated in the presence of microsomes and the location of the epitope in the microsomal membrane was examined by protease sensitivity. Their results indicated that the C-terminus was intracellular while two positions just upstream and down stream of the flip/flop region as well as both N- and C-terminal sides of the TM2 were extracellular. This study indicates that at least part of the M3-M4 is extracellular and that TM2 does not cross the membrane since both ends of TM2 are intracellular (Figure 1).

Several other studies have confirmed that the pore-forming region, TM2, does not cross the membrane but forms a hairpin loop structure within the membrane. Domain deletion in
GluR1 (Hollmann et al. 1994) and gold fish kainate binding proteins, GFKARs (Wo and Oswald, 1995, 1996) have been used to demonstrate that TM2 does not cross the membrane. Moreover, Kuner et al. (1996) have investigated the structure of the TM2 segment in NMDA receptor by assessing the accessibility pattern of substituted cysteine residues. They probed the substituted cysteine residues on the extracellular and cytoplasmic faces of NR1/NR2C subunits with charged sulfhydryl-specific reagents and showed that the TM2 segment forms a hairpin-loop within the membrane. Thus, a membrane topology with 3TM domains (TM1, TM3, and TM4) and a membrane segment that forms a hairpin loop (M2) has been proposed. (Figure 1; Hollmann et al. 1994; Wo and Oswald, 1994).

An intracellular C-terminus and a hairpin loop-forming membrane segment, M2, would mean that the M3-M4 loop is located extracellularly. Several studies have used N-glycosylation as a topological marker to show extracellular localization of the M3-M4 loop. This loop has been shown to contain a naturally glycosylated asparagine (N) residue in GluR6 receptors, and mutagenic removal of the residue results in loss of glycosylation (Roche et al. 1994; Taverna et al. 1994). Also, analysis of native N-glycosylation sites in the M3-M4 loop of two kainate binding proteins obtained from gold fish, GFKARα and GFKARβ, indicated that these site were glycosylated in microsome-supplemented in vitro translation system (Wo and Oswald, 1994, 1995). Moreover, Hollmann et al. (1994) have introduced several N-glycosylation sites at various positions from the N-terminal to the C-terminal domain throughout GluR1 receptors and assessed glycosylation of these sites. In this study, all four sites introduced in the N-terminal domain were glycosylated, but no glycosylation was observed for the six sites introduced in the TM2 and surrounding regions. Moreover, seven of eight sites in the M3-M4 loop but none of
three sites in the C-terminal domain were glycosylated. These results indicated that while the C-terminus is intracellular, the N-terminus and M3-M4 loop are extracellular.

More topological evidence for an extracellular location of the M3-M4 loop comes from studies of ligand binding sites and receptor desensitization. The observation that two non-continuous regions in iGluRs, ~130 amino acids just N-terminal to TM1 (S1 segment) and the M3-M4 loop (S2 segment) share high sequence homology with bacterial glutamine-binding protein, QBP (O’Hara et al. 1990) provided the first clue in the search for the ligand binding site in iGluRs. Several studies have provided evidence for the involvement of amino acids within the S2 segments in receptor desensitization and agonist binding. Sommer et al. (1990) have shown that an alternatively spliced exonic sequence of ~38 amino acids just N-terminal to the TM4 domain of AMPA receptors, the flip/flop region, affects desensitization of the channels. In addition, Lomeli et al. (1994) have identified an RNA-editing site immediately preceding the flip/flop region of AMPA receptors, R/G site, that affects recovery from desensitization.

Further studies by Stern-Bach et al. (1994) provided evidence for the involvement of S1 and S2 segment in agonist binding and receptor desensitization. These workers made chimeric proteins by exchanging the S1 and S2 segments of GluR3 with that of GluR6 and showed that agonist selectivity of these receptors is specified by amino acid residues in both S1 and S2 segments. In this study, homologous exchange of the S1 and S2 segments altered the pharmacological profile of the channel such that the recipient subunit assumed the agonist binding and desensitization properties of the donor subunit. In another study, Swanson et al. (1997) mutated several amino acid residues in the S2 segment of GluR6 to the GluR5 counterparts and showed that amino acid residues within the S2 segment are involved in agonist binding and gating activity of the channels. In this study, mutation of residues in the S2 segment
of GluR6 receptor altered AMPA sensitivity, domoate deactivation, and kainate desensitization of the channel.

Several studies have used active phosphorylation sites to provide topological evidence for iGluRs. Raymond et al. (1993) have shown that GluR6 receptors expressed in mammalian cells are phosphorylated by protein kinase A (PKA). They proposed the Ser 684 residue, located within the M3-M4 loop, as a PKA consensus site. In this study, intracellular perfusion of GluR6-expressing cells with the catalytic subunit of PKA potentiated glutamate-induced currents. Moreover, mutation of serine to alanine (S684A) eliminated PKA-mediated phosphorylation and potentiation of glutamate-induced currents. In addition, Wang et al. (1993) have shown that mutation of two serine residues located within the M3-M4 loop of GluR6, Ser 684 and Ser 666, abolished PKA potentiation of kainate-induced currents. They suggested Ser 684 as the preferred site of PKA-phosphorylation in native GluR6 receptors.

Other studies have reported phosphorylation and potentiation of kainate induced currents in GluR1 and GluR6 receptors by Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) and by PKC (McGlade-McCulloh et al. 1993). The site of phosphorylation was proposed to be Ser 627 in GluR1 since mutation of this residue to alanine (S627A) eliminated CaMKII phosphorylation and potentiation of the currents (Yakel et al. 1995). Moreover, Nakazawa et al. (1995) have studied phosphorylation of AMPA receptors in cerebellar Purkinje cells using antibodies that recognize a stretch of amino acids within the M3-M4 loop phosphorylated at Ser 696 of GluR2 or homologous sites in GluR1, GluR3, and GluR4 receptors. Their results indicated that Ser 696 in GluR2 or the corresponding sites in other AMPA receptors were phosphorylated upon activation of the receptors. The results of these studies contradict an extracellular location for the M3-M4 loop since phosphorylation of the receptors normally occurs at the cytoplasmic side.
1.7. Role of cysteine residues in the function and pharmacological properties of iGluR.

The structure and function of ligand gated ion channels are highly dependent on conditions affecting the redox state of cysteine residues (Karlin and Bartels 1966; Lipton et al. 1993; Gozlan and Ben-Ari 1995). Both reducing and oxidizing agents have been shown to modulate the agonist affinity as well as channel properties of several ligand-gated ion channels including acetylcholine, GABA, glycine and glutamate receptors as well as gold fish kainate binding proteins (GFKARs) (Karlin and Bartels 1966; Aizenman et al. 1989; Köhr et al. 1994; Pan et al. 1995; Wo and Oswald 1996).

Selective modulation of NMDA-mediated responses by both endogenous and exogenous redox agents has been described in various neuronal preparations as well as non-neuronal cells expressing recombinant NMDA receptors (Terramani et al. 1988; Aizenman et al. 1989 & 1990, Lei et al. 1992; Tang and Aizenman 1993a & 1993b; Gozlan et al. 1994; Köhr et al. 1994; Gozlan and Ben-Ari 1995). The redox modulation of iGluRs is thought to have both neuroprotective and neurodestructive effects (Lipton et al. 1993). Thus, redox agents have been proposed as potential therapeutics for neuropathological conditions (Lipton et al. 1993; Hammond et al. 1994; Gozlan and Ben Ari, 1995).

Using site-directed mutagenesis, homologous pairs of cysteine residues that presumably form disulfide bonds have been identified as the target sites for redox sensitivity of NMDA and GFKAR (Sullivan et al. 1994; Wo and Oswald 1996). These sites (Cys726 and Cys780 in NR1, and Cys305 and Cys358 in GFKARβ) are well conserved in all iGluRs (Figure 18). Despite this conservation, no modulatory effect of redox reagents such as dithiothreitol (DTT) or 5,5'
dithiobis-2-nitrobenzoic acid (DTNB) has been observed for the non-NMDA receptors (Terramani et al. 1988; Aizenman et al. 1989), although chemical modification of thiol group(s) affected the function of AMPA/KA receptors (Kiskin et al. 1986; Terramani et al. 1988; Basiry et al. 1999; see results, section 3.1.4).

No direct evidence indicating the presence of disulfide bond(s) in intact AMPA/KA receptors exists. However, in a recent study by Armstrong et al. (1998) the crystallized structure of the ligand-binding domain of GluR2 (i.e. the S1-S2 segment connected by a linker) revealed a disulfide bond between Cys718 and Cys773. In addition, experiments on the S1-S2 segment of GluRD (GluR4), suggest the existence of a disulfide bond between corresponding Cys719 and Cys774 (Abele et al. 1998). Moreover, a change in the kinetic properties of the GluR3 Cys722 mutant (Watase et al. 1997) indicates that this cysteine residue is involved in channel gating, perhaps through disulfide-bond formation. In GluR3, mutation of Cys722 to alanine (C722A) resulted in a higher affinity for glutamate or kainate, and enhanced receptor sensitivity to aniracetam (Watase et al. 1997) which slows deactivation and desensitization of AMPA receptors (Partin et al. 1996). Taken together, the aforementioned data indicate that extracellular cysteine residues, in their thiol or disulfide-bond states, may play an important role in the structure and function of iGluRs.

**1.8. Research Hypothesis.**

Two possible explanations for the conflicting data for the N-terminal half of the AMPA/KA receptor M3-M4 loop include: 1) there are two additional transmembrane segments
in this region; 2) the M3-M4 loop is dynamic, that is this structure is located extracellularly in
the receptor's unliganded state but undergoes membrane translocation upon agonist binding and
channel gating. Both explanations seem energetically implausible, since there are no sustained
segments of hydrophobic residues in this region (Asn 623 to ~Thr 710 in GluR6). Moreover,
models of iGluRs based on the known structure of the highly homologous bacterial
lysine/arginine/ornithine-binding protein, LAOBP (Wo and Oswald, 1994; Stern-Bach et al.,
1994; Sutcliffe et al., 1996), do not support the first explanation. In favor of the second
explanation, gating-associated membrane translocation of polar and even charged residues has
been demonstrated for some voltage-gated channels (Slatin et al., 1994; Larsson et al., 1996).

Here, we tested the possibility that the putative target for PKA phosphorylation, Ser 684
of GluR6, undergoes membrane translocation upon agonist binding. Cysteine-specific
biotinylation of wild-type and mutant (S684C) GluR6 was compared using biochemical methods,
and functional consequences were assessed by patch clamp recording.

In addition, we examined the role of cysteine residues located on the M3-M4 loop as well
as on the extracellular N-terminal region on the function and pharmacological properties of
homomeric GluR6 receptors. We mutated putative extracellular cysteine residues to serine or
threonine to examine formation of disulfide bonds by these residues as well as their contribution
to the function of homomeric GluR6 receptors expressed in HEK-293 cells. The results of these
studies, presented in this thesis, increase understanding of the structural determinant of KAR
function.
CHAPTER 2

MATERIALS AND METHODS


The cDNA encoding rat GluR6 was subcloned into a mammalian expression vector containing the cytomegalovirus promoter, as previously described (Raymond et al., 1993). Site-directed mutagenesis was performed using the Stratagene Chameleon Kit. To generate the Ser to Cys mutation at position 684 (S684C; amino acid numbering based on mature protein), we used 5' -CAG GAG ACA GTG TGT CTA AAG CAA TGA GG- 3' as the mutagenesis primer and 5' -AGA GGA ACT TGG TTA GGG CCC TTC TGA GGC GGA AAG AAC- 3' as the selection primer, converting a KpnI to an Apal restriction site in the vector.

For the construction of C65S, C199S, C316S, C432S, C719S, C719T, C773 and C719/C773S the following primers were used: C65S, 5' -TCT AAG AAA GCT TCT GAT CAG CTG TCT CTT GGG GTG- 3'; C199S, 5' -GTG ATC TTC GAC TCC AGC CAT GAG ATG GCA GCA GGC - 3'; C316S, 5' -AGC TCC TTG CAA TCC AAT CGA CAC AAA CCC TGG CGC- 3'; C432S, 5' -TTT GAA GGC TAC TCT ATT GAT CTC CTA CGA GAG TTA- 3'; C719S and C719S/C773S, 5' -TAC ACA GCG GAA CTC TAA CCT CAC GCA GAT TGG- 3'; C719T, 5'-ACA CAG CGG AAC ACT AAC CTC ACG CTA CGA GAG TTA- 3'; C773S, 5' -GGT GGC GGG GCA ATG GCT CCC AAT CAT GAG GGC GCA AAG AGG- 3' as the mutagenic primers and 5' -GAG GAA CTT GGT TAG GCC CCT TCT GCT GAG GCG AAG AGA ACC- 3' as the selection primer. For the double mutant (C719S/C773S) 5' -AGA GGA ACT
TGG TTA GGT ACC TTC TGA GGC GGA AAG AAC- 3' was used as the selection primer. Restriction analysis and standard sequencing methods confirmed the mutations.

2.2. Transient transfection of wild-type and mutant GluR6 in HEK 293 cells.

Human embryonic kidney 293 (HEK 293) cells from American Type Culture Collection (CRL 1573) were cultured as previously described (Raymond et al., 1996). The cells were passaged every 2-3 days and plated at a density of 1-2 ×10^6 cells/ml 10-14 hours before transfection. Cells were plated directly onto poly-D-lysine (10 µg/ml) coated culture dishes in preparation for biochemical experiments. The cells were transiently transfected with cDNA encoding wt or mutant GluR6 (10 µg plasmid / 10 cm plate) using calcium-phosphate coprecipitation. For the transfection, the cDNA was first dissolved in a solution containing the following reagents: 450µl of 1xTE (Tris.Cl, 10mM / Ethylenediaminetetraacetic acid or EDTA, 1mM); 500µl of 2xBES, pH6.96 (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 50 mM / NaCl, 280 mM / Na_2HPO_4·2H_2O, 1.5 mM), and 50µl of 2.5 M CaCl_2. The solution containing cDNA was then placed in the hood for ~20 minutes before adding it to the cells. The cells were then placed in a 3% CO_2 incubator for ~10-12 hours. At the end of this incubation time, the cells were washed twice with warm phosphate-buffered saline (PBS) to terminate the transfection and 10 ml of fresh medium was added to the cells. Finally, the cells were incubated in a 5% CO_2, 37°C incubator for another 36 hours before biochemical analysis.
2.3. Biotinylation of wt and mutant GluR6 and preparation of samples.

Biochemical analysis was performed 48 hours following transfection. Transfected cells were washed twice with warm PBS solution and incubated at 37°C and 5% CO₂ for 30-60 min with either N-hydroxysuccinimide-SS-Biotin (NHS-SS-Biotin; 1 mg / ml) or N-[6-(biotinamido)hexyl]-3’-(2’-pyridyldithio)propionamide (HPDP-Biotin; 0.03 mg / ml) in serum-free medium or in balanced saline solution (Hall et al., 1997). Cells were then washed 5 - 6 times with warm PBS to remove the biotinylating reagent. Isolation of the membrane fraction and precipitation of biotinylated proteins were performed essentially as described elsewhere (Hall et al., 1997). Briefly, the cells were collected in ice-cold harvest buffer containing 1 mM EDTA, 40 units/ml aprotinin (Trasylol), and 1 mM phenylmethylsulfonyl fluoride (PMSF) in PBS (pH 7.4); lysed by 15 s sonication; and centrifuged for 20 min at 4 °C, 14,000 rpm (Eppendorf Microcentrifuge 5415C). Membrane proteins were isolated by resuspending the pellet in 1 ml of harvest buffer containing 1% Triton X-100, centrifuging the suspension (5 min, 5000 rpm, 4 °C), and collecting the supernatant.

Aliquots of the membrane preparation, ranging from 10 - 80 μl and corresponding to 1 - 8% of total protein, were reserved for loading on SDS-PAGE. The rest of the membrane preparation was incubated end-over-end with ~100 μl streptavidin-linked beads at 4 °C for 2 hours. Beads were collected by brief centrifugation and washed extensively with 1% Triton X-100 in harvest buffer. Bead-precipitated protein was eluted by end-over-end incubation in 100-200 μl gel loading buffer (containing 150 mM dithiothreitol -- DTT) at 4 °C for 30 min followed by brief centrifugation.
2.4. Western blot analysis of wt and mutant GluR6.

The supernatant, together with the aliquots of the total membrane preparation, were subjected to 8% SDS-PAGE. The gel running buffer contained glycine (1.90M), Tris (0.25M), and sodium dodecyl sulfate (SDS, 0.035M). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in a transfer buffer (Tris 1M, glycine 0.1M, and methanol 20%) and immunoblotted using affinity purified anti-GluR6/7 polyclonal antibodies (0.7 μg/ml). In the blotting procedure, the membrane was incubated for 2 hours at room temperature with tris-buffered saline-tween (TBS-T, 0.1% Tween-20) solution containing 5% powdered milk. Following two washes with TBS-T (2x10 min), the membrane was incubated with the primary antibody for 1 hour, with agitation at room temperature. After removing the primary antibody, the membrane was washed with the TBS-T solution (3x10 min) and incubated with a secondary antibody (Donkey anti-rabbit immunoglobulin, horse radish peroxidase linked) for 30 min with agitation, at room temperature. The membrane was then washed with TBS-T solution (4x10 min) and incubated with enhanced chemiluminescence (ECL) reagent for 1 min. The protein bands were visualized by exposing the membrane to films in dark room.

2.5. Quantitation and construction of standard curve for wt and mutant GluR6.

Protein bands were quantitated by densitometry, and a standard curve was constructed using measurements made from the lanes containing 1, 2, 4, and 8% of the total membrane protein. This curve was used to quantitate the amount of bead-eluted GluR6, corresponding to biotinylated GluR6, as a percentage of total membrane protein. The amount of bead eluted
protein was calculated using the equation \( Y = mX + b \) where \( Y \) is the density of the “bead” protein band, \( X \) is the percent of total protein in bead fraction (or bead eluted protein), \( m \) is the slope of the standard curve, and \( b \) is the \( y \)-intercept.

2.6. Electrophysiology.

Immediately following transfection, cells for patch clamp recording were replated into 35 mm culture dishes containing glass cover slips. 48 - 80 hours following transfection, the cells were transferred on a glass coverslip to the stage of an inverted microscope (Axiovert 100, Carl Zeiss, Thornburg, NY). Patch clamp recordings (Hamill et al., 1981) were made at room temperature (20-22 °C). Currents were sampled at 10 KHz, filtered at 5 KHz, and acquired and analyzed using pCLAMP6 software and the Axopatch 200A amplifier (Axon Instruments, Foster City, CA).

Electrodes were fabricated from 1.5 mm outer diameter thin wall borosilicate glass (Warner Instrument Corp, Hamden, CT) using the Narishige PP-83 vertical puller (Narishige Scientific Instruments, Tokyo, Japan). Electrode resistance ranged from 2 - 5 MΩ when filled with solution containing: 145 mM KCl, 5.5 mM 1,2-bis(2-aminophenoxy)ethane-\( N,N,N',N' \)-tetraacetic acid (BAPTA), 0.5 mM CaCl\(_2\), 2 mM MgCl\(_2\), 2 mM tetraethylammonium chloride, 4 mM MgATP, and 10 mM HEPES (pH 7.2).

Cells were continuously superfused with extracellular recording solution containing: 145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 11 mM glucose, and 10 mM HEPES (pH 7.35). Immediately following seal formation, each cell was lifted from the floor of the recording chamber and placed within 100 μm of the tip of a theta tube (Hilgenberg, Malsfeld, Germany;
each barrel tip had an inner diameter of ~200 μm). Cells with membrane capacitance ranging from 8 - 16 pF were chosen for recording. Control and agonist solutions were continuously gravity-fed through the two sides of the theta tube. Rapid exchange between these two solutions was accomplished by a computer-triggered piezo-electric device (Physik Instruments, Waldbronn, Germany), as previously described (Chen et al., 1997). The 10-90% rise time for exchange of the two solutions at the open tip of the recording electrode was <0.5 msec. Agonist was applied for 100 ms at 60 s intervals to monitor the peak current amplitude and desensitization time constant. Cells were exposed to 0.03 mg/ml HPDP-Biotin (with or without 1 mM kainate) or 0.01 mg/ml purified streptavidin by continuous superfusion through the control side of the theta tube. Application of HPDP-Biotin was begun only after a stable current response was established (~5-6 min following initial agonist application).

2.7. Data Analysis.

Current recordings were stored on hard disk for later analysis by pCLAMP6 Clampfit software, using a Pentium 90 MHz personal computer. Desensitization time constants were determined by adjusting cursors in Clampfit to find the best (visual) fit of the current decay to a single exponential function, using the Chebyshev method; peak current was taken to be the amplitude extrapolated to the first cursor, set at the peak by the Clampfit program. Curve-fitting for dose-response data and the generation of standard curves from densitometry measurements were accomplished with Origin or Excel software, respectively. All values are shown as mean ± S.E.M., unless otherwise indicated. Statistical comparisons were made using the two-tailed Student’s t test, either paired or unpaired (as specified), with a 95% confidence limit.

NHS-SS-Biotin was dissolved into the experimental solution at 1 mg/ml just before use. HPDP-Biotin was made up as a 3 mg/ml stock solution in dimethylsulfoxide (DMSO) and kept at 4 °C. 1 mg/ml streptavidin stock solutions were made up in extracellular recording solution on the day of use and kept on ice. For patch clamp recording experiments, HPDP-Biotin and streptavidin stocks were diluted 100-fold into extracellular recording solution just before addition to the cells. Stock solutions of kainate and glutamate (100 mM and 1 M, respectively), as well as of CNQX (20 mM in DMSO), were maintained at -20 °C and thawed only once.

Streptavidin-linked beads, HPDP-Biotin, and sulfo-NHS-SS-Biotin were from Pierce (Rockford, Illinois). Purified streptavidin was from Molecular Probes (Eugene, Oregon). CNQX was from RBI (Natick, MA). Culture media and reagents were from Canadian Life Technologies/Gibco BRL (Burlington, ON). PVDF membranes and SDS-PAGE reagents were from Bio-rad Laboratories (Hercules, CA). ECL reagents were from Amersham (Buckinghamshire, England). Anti-GluR6/7 polyclonal antibodies were either gifts from Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD) or purchased from Upstate Biotechnology. All other reagents were from Sigma (St. Louis, MO).
CHAPTER 3

RESULTS

SECTION I: Structure of M3-M4 loop

3.1.1. Increased amount of cysteine-specific biotinylation of GluR6(S684C) compared with wt GluR6.

In a previous study, Slatin et al. (1994) used cysteine substitution followed by cysteine-specific biotinylation of the bacterial channel, colicin IA, to show that voltage-dependent transitions to the open states could be prevented by addition of streptavidin to the cis side, and transition to the closed state prevented by addition of streptavidin to the trans side of a lipid bilayer. Therefore, to investigate the location of Ser 684 in the agonist bound and unbound state of GluR6, we first mutated this residue to Cys to allow modification of this site by Cys-specific biotinylating reagents and streptavidin. Importantly for our study, whole-cell patch clamp recordings made from HEK 293 cells expressing the mutant GluR6(S684C) exhibited 1 mM glutamate (GLU)-evoked current responses similar to those recorded from wt GluR6-transfected cells (e.g., Fig. 13). Furthermore, the GLU dose-response curves for these two receptors were nearly identical (Fig. 2), as were other macroscopic properties of the GLU-evoked currents (Table 1).

To test whether Cys 684 is accessible to modification from the extracellular side of the cell membrane, we briefly incubated live GluR6(S684C)- or wt GluR6-transfected cells with the Cys-specific reagent HPDP-Biotin (Slatin et al., 1994). Western blot analysis with GluR6-
specific antibodies was used to compare the amount of biotinylated receptor recovered following incubation with streptavidin (SA)-linked beads. In addition, in order to measure total \textit{wt} GluR6 and GluR6(S684C) surface receptor expression, we used extracellular NHS-SS-Biotin, which targets primary amines, so that all surface receptors would be expected to incorporate at least one biotin and thus be recovered by SA-linked bead precipitation.

Using NHS-SS-biotin, surface expression of \textit{wt} GluR6 and GluR6(S684C) were very similar: \(4.8 \pm 0.3\%\) (n=11) and \(4.1 \pm 0.5\%\) (n=7) of total receptor in the cell lysate, respectively (Fig. 3, 4 and 5). On the other hand, \textit{wt} GluR6 recovered with SA-linked beads following incubation with HPDP-Biotin was only \(2.5 \pm 0.5\%\) (n=11), or \(~50\%\) of that recovered after incubation with NHS-SS-Biotin (Fig. 3 and 5; significant difference by unpaired \(t\) test, \(p=0.0004\)). Diminished recovery after HPDP-Biotin is not surprising, since \textit{wt} GluR6 contains just seven putative extracellular Cys residues, and only those which are in the reduced state and relatively exposed to aqueous solution (i.e. not buried within a globular protein domain) are available for modification by HPDP-Biotin. In contrast, SA-linked bead recovery of GluR6(S684C) after incubation with HPDP-Biotin was \(4.5 \pm 0.8\%\) (n=12) (Fig. 3 and 5), which was significantly higher than that of \textit{wt} GluR6 (\(p=0.04\), unpaired \(t\) test) and not significantly different from the recovery of GluR6(S684C) after treatment with NHS-SS-Biotin (\(p=0.699\), unpaired \(t\) test).

As a control to confirm that extracellularly applied HPDP-Biotin only modifies extracellular Cys residues, we used the same protocol to measure recovery of the cytoplasmic enzyme, microtubule-associated protein kinase (MAPK), by SA-linked bead precipitation. Western blot analysis with antibodies specific for MAPK showed no signal in the bead-
precipitated fraction in spite of a robust signal for the cell lysate (not shown), confirming that there was no significant biotinylation of MAPK by extracellularly applied HPDP-Biotin. Taken together, these data suggest that Cys 684 is freely accessible to aqueous solution (and HPDP-Biotin) on the extracellular side of the membrane.

3.1.2. Decreased accessibility of Cys 684 to biotinylation following agonist binding.

To determine whether Cys 684 remains extracellular and accessible to modification when the receptor is in the ligand-bound state we compared SA-linked bead recovery of \( \text{wt} \) GluR6 and GluR6(S684C) after incubation with HPDP-Biotin in the presence vs. absence of a saturating concentration of agonist. For \( \text{wt} \) GluR6, there was a small increase in SA-linked bead recovery when 1 mM kainic acid (KA) was included during incubation with HPDP-Biotin (Fig. 6, 7, and 8; Ratio +KA/-KA \( \approx 1.25 \)) but this trend was not significant (N=8; \( p=0.10 \), paired \( t \) test). In contrast, recovery of GluR6(S684C) decreased significantly (N=9; \( p=0.031 \), paired \( t \) test), to approximately the same level as seen for \( \text{wt} \) GluR6, when HPDPrBiotin incubation was performed in the presence of 1 mM KA (Fig. 6 and 8). The difference between \( \text{wt} \) GluR6 and GluR6(S684C) in the ratio of receptor recovered by SA-linked beads after incubation with HPDP-Biotin in the presence of 1 mM KA to that recovered under the control condition (HPDP-Biotin incubation without KA) for each of the experiments was highly significant (p=0.007, unpaired \( t \) test; Fig. 8). These data strongly suggest that the conformational change in GluR6(S684C) that is associated with KA binding renders Cys 684 inaccessible to modification by HPDP-Biotin.
It is possible that mutation of Ser at position 684 results in a conformational change in the unliganded receptor, such that distant Cys residues become more accessible to aqueous solution (and HPDP-Biotin), and that the conformation of ligand-bound GluR6(S684C) reverts to that of ligand-bound \textit{wt} GluR6. To test whether substitution of an amino acid other than Cys at position 684 results in comparable SA-linked bead recovery of receptors after HPDP-Biotin vs. NHS-SS-Biotin, as well as significantly decreased SA-linked bead recovery after incubation with HPDP-Biotin in the presence vs. absence of agonist, we repeated the same experiments with GluR6(S684A). This mutant receptor also shows current responses and sensitivity to glutamate similar to \textit{wt} GluR6 (Raymond et al., 1993).

Like \textit{wt} GluR6, we found that SA-linked bead recovery of GluR6(S684A) after incubation with HPDP-Biotin was markedly lower than that recovered following treatment with NHS-SS-Biotin (ratio of 0.57 ± 0.12, N=3). Moreover, SA-linked bead recovery of the mutant GluR6(S684A) after HPDP-Biotin incubation in the presence vs. absence of 1 mM kainate was not significantly different (ratio of 1.01 ± 0.06, N=4; p=0.656, paired \textit{t} test), again similar to that observed for \textit{wt} GluR6. These data indicate that mutation of Ser 684 alone (i.e., to an amino acid other than Cys) is not sufficient to alter biotinylation of Cys-residue elsewhere in the protein. These results, together with the fact that GluR6(S684C) current responses and sensitivity to glutamate are similar to that of \textit{wt} GluR6, support the conclusion that the substituted Cys at 684 is biotinylated by HPDP-Biotin and that accessibility of this reagent to Cys 684 is markedly decreased in the presence of kainate.
3.1.3. Agonist-induced conformational change alters accessibility of Cys 684 to extracellular reagent.

The decrease in accessibility of Cys 684 to extracellular HPDP-Biotin observed in the presence of 1 mM KA could be due to: 1) agonist-induced movement of residue 684 to a position less exposed to aqueous solution on the extracellular side of the membrane; 2) agonist-induced membrane translocation of this residue; or 3) the fact that occupation of the KA-binding site itself blocks access to Cys 684. Previous studies have indicated that different agonists and competitive antagonists coordinate with different amino acids within the binding pocket of GluRs (Paas et al., 1996; Swanson et al., 1997).

In order to test the third possibility, we compared SA-linked bead recovery of GluR6(S684C) after HPDP-Biotin incubation in the presence vs. absence of another agonist, glutamate, or the competitive antagonist, CNQX (Honore et al., 1988). Results for HPDP-Biotin incubation in the presence vs. absence of 1 mM glutamate are shown in Figures 9 and 12. Similar to results of experiments with kainate, there was a marked decrease in Cys-specific biotinylation of GluR6(S684C) but little change in that of wt GluR6 when HPDP-Biotin incubation was carried out in the presence of 1 mM glutamate (significant difference between ratios for wt and mutant GluR6 by unpaired t test, p=0.022; also compare Figs. 8 and 12). On the other hand, in the presence of a nearly saturating concentration of CNQX (10 μM; Wilding and Huettner, 1996), there was little change in HPDP-biotinylation of GluR6(S684C) compared with the absence of CNQX (Fig. 9 and 12), and the ratio of recovery of biotinylated GluR6(S684C) in the presence vs. absence of CNQX was significantly different from the ratio in the presence vs. absence of glutamate or kainate (p=0.016 and 0.027, respectively, unpaired t test). Apparently,
both occupation of the ligand binding site and channel gating are required in order for Cys 684 to become inaccessible to extracellular HPDP-Biotin.

3.1.4. Cys-specific biotinylation alters peak current amplitude and desensitization of both wt and mutant GluR6.

To determine whether Cys 684 in GluR6(S684C) remains extracellular (though buried), or if it actually translocates to the cytoplasmic side of the membrane upon agonist binding and channel activation, we performed whole-cell patch clamp recording. We compared the current response evoked by 1 mM glutamate recorded from GluR6(S684C)- vs. wt GluR6-transfected cells after incubation with HPDP-Biotin followed by streptavidin. We assumed that any differences observed in the current response mediated by GluR6(S684C) compared with that of wt GluR6 would be due to modification of Cys 684.

As illustrated in Figure 13, incubation for ~15 minutes with HPDP-Biotin, followed by 6-8 minutes of washout, had a similar effect on GluR6(S684C)- and wt GluR6-mediated currents. In both cases, we observed a significant, irreversible decrease of 1 mM glutamate-evoked peak current amplitude along with slowing of onset of agonist-induced desensitization ($\tau_D$), although the latter was not significant due to high variability in the extent of slowing. In contrast, a 15 minute incubation with vehicle alone (1% DMSO) resulted in a smaller decrease in peak current amplitude and no change in $\tau_D$ (Fig. 14 and 15). Thus, these alterations in macroscopic current would be consistent with effects of biotinylation of Cys residue(s) present in both wt GluR6 and GluR6(S684C). From these results, we concluded that if Cys 684 was modified by HPDP-Biotin
under conditions used in these patch clamp recording experiments, there was no functional effect of the addition of biotin to this residue. As a further test of this conclusion, we analyzed the effect of incubating GluR6(S684C)-transfected cells with HPDP-Biotin in the presence of 1 mM kainate. As discussed above, analysis by Western blot suggested that inclusion of 1 mM kainate along with HPDP-Biotin prevented biotinylation of Cys 684 (Figure 6). Consistent with the conclusion that biotinylation of Cys 684 is functionally "silent", we found no significant difference in effects on GluR6(S684C) peak current amplitude or $\tau_D$ after incubation with HPDP-Biotin in the presence vs. absence of 1 mM kainate (Fig. 14 and 15).

3.1.5. Treatment of Cys-biotinylated GluR6(S684C) with extracellular streptavidin leaves channel activation intact but slows desensitization.

Next we analyzed recordings made from $wt$ GluR6- and GluR6(S684C)-transfected cells following biotinylation and extensive washout of HPDP-Biotin and then incubation with extracellular streptavidin. In contrast to HPDP-Biotin, the addition of streptavidin had no significant effect on peak current amplitude for either $wt$ GluR6 or GluR6(S684C) (Figure 16 and 17). On the other hand, there was a further significant slowing of onset of agonist-induced desensitization seen for GluR6(S684C)-mediated, but not $wt$ GluR6-mediated, currents after treatment with streptavidin (Figure 16 and 18). Moreover, if 1 mM kainate was included during the incubation with HPDP-Biotin, streptavidin had no effect on the rate of macroscopic desensitization of GluR6(S684C)-mediated current (Figure 16 and 18). Since an effect of streptavidin was seen for GluR6(S684C) and not for $wt$ GluR6 after incubation with HPDP-Biotin in the absence of agonist, we conclude that Cys 684 in GluR6(S684C) is biotinylated by
HPDP-Biotin and accessible to streptavidin from the extracellular side of the membrane, a result consistent with our biochemical evidence (see above). However, since streptavidin had no effect on peak current amplitude and only a small effect on macroscopic desensitization, it is unlikely that Cys 684 undergoes membrane translocation upon agonist binding and channel activation.

Taken together, the results of both biochemical and patch clamp recording experiments suggest that the amino acid residue at position 684 in GluR6 is extracellular in the receptor’s unliganded state as well as in the ligand-bound, activated state.
RESULTS

SECTION II: Role of cysteine residues in the structure and function of GluR6 receptor

3.2.1. Assessment of the role of Cys-residues located on the M3-M4 loop and N-terminal domain of GluR6 receptors

GluR6 receptors contain two Cys-residues located in the M3-M4 loop and five Cys-residues in the N-terminus. We have mutated several of these residues to serine or threonine to determine whether they participate in formation of disulfide bonds and to assess their role in the function of the GluR6 receptor channel. We first assessed protein expression of the mutant receptors using Western blot analysis. Protein expression was observed for the mutant GluR6 receptors C719T, C719S, C199S, and C65S. However, several mutations including C773S, C316S, and double mutation C719S/C773S resulted in loss of protein expression in transfected HEK-293 cells (Table 2).

In order to examine if these Cys-residues form disulfide bonds we performed HPDP-Biotin analysis of these mutant receptors. For protein expression, similar to the protocol described for GluR6(S684C) and wt GluR6 receptors, we incubated HEK-293 cells transfected with GluR6(C719T), GluR6(C199S), and GluR6(C65S) as well as wt GluR6 with the Cys-specific reagent HPDP-Biotin. Then, we compared the amount of biotinylated receptors recovered following incubation with streptavidin (SA)-linked beads using Western blot analysis followed by densitometry (Fig. 20 and 23). If a Cys-residue is involved in a disulfide bond, we predict that mutation of this residue would free a second Cys-residue. If the second Cys-residue
is readily accessible to the extracellular solution, the amount of HPDP-biotin recovery of the protein should be significantly increased. We obtained some preliminary data on the involvement of Cys719 and Cys65 in disulfide bond formation as well as their role in the function of GluR6 receptors.

3.2.2. Increased amount of cys-specific biotinylation of GluR6(C719T) compared with wt GluR6.

Western blot analysis of GluR6(C719T) indicates a significantly increased amount of HPDP-biotinylation and SA precipitation of this mutant compared with wtGluR6 (N=3; P < 0.001; Fig 20 and 21) suggesting that this residue is involved in a disulfide bond. Figure 20 illustrates a representative Western blot of GluR6(C719T) and wt GluR6 receptors. A similar result was obtained for GluR6(C719S).

3.2.3. C719T mutation affects desensitization time course of glutamate-evoked currents.

Whole-cell patch clamp recordings from wt GluR6- and GluR6(C719T)- expressing HEK-293 cells were made under voltage-clamp (Vh = -60mV). Analysis of current evoked by a 100 ms application of a saturating concentration of glutamate (10 mM) showed no difference in the mean rise time (10-90% current activation) for GluR6(C719T) compared with wt GluR6 (2.21 ± 0.25 ms, n=7, and 2.08 ± 0.18 ms, n=8, respectively). However, the desensitization time course of glutamate-evoked currents was prolonged for GluR6(C719T) compared to wt GluR6. The desensitization time constant (τD) for GluR6(C719T) was 15.7 ± 1.1 ms (n=7), ~2.6-fold.
slower than that of wt GluR6 receptors (6.0 ± 0.3, n=8). Representative current traces for GluR6(C719T) and wt GluR6 are illustrated in Figure 22.

3.2.4. Increased amount of Cys-specific biotinylation of GluR6(C65S) but not GluR6(C199S) compared with wt GluR6.

Mutation of Cys-65 to Ser (C65S) resulted in a significant increase in HPDP-biotin-SA precipitation of the receptor (N=4; P < 0.005; Fig 23 and 24). Mutation of Cys199 to Ser, however, did not result in a significant change in Cys-specific biotinylation of the receptor (N=4; Figure 23 and 24). Figure 23 illustrates representative Western blot of C65S and C199S. Biotinylation of GluR6(C65S) was approximately 8-fold higher than wt GluR6 (Figure 24). These data suggest that Cys65 is involved in a disulfide bond.
CHAPTER 4

Figures and Figure Legends
Figure 1: Current transmembrane topology of iGluRs with 3 transmembrane domains (TMD 1, 3, and 4) and one reentrant TM2, extracellular N-terminus and M3-M4 loop (S2 segment) and intracellular C-terminus. The approximate position of Ser 684 and extracellular cysteine residues (C) are shown. Amino acid numberings based on mature protein.
Figure 2. Glutamate dose-response curves for wt GluR6 and GluR6(S684C) are similar. Peak glutamate-evoked current was recorded from cells transfected with either wt GluR6 (■) or GluR6(S684C) (○), and the amplitude (I) was normalized to the peak current response to 10 mM glutamate ($I_{\text{max}}$) for each cell. Points represent data from N=3-9 different cells. Curves were fitted to the equation $I = I_{\text{max}} \times \left(1/(1 + (EC_{50}/[GLU])^n_{H})\right)$, where $n_H$ is the Hill coefficient. The $EC_{50}$ and $n_H$ values were: 330 ± 20 µM and 1.1 for wt GluR6, and 320 ± 10 µM and 1.0 for GluR6(S684C). The data were collected and analyzed by Dr Raymond.
Table 1: Comparison of macroscopic current properties of wt and mutant GluR6.

<table>
<thead>
<tr>
<th></th>
<th>( \tau_D^* ) (msec)</th>
<th>Rise-time(^&amp;) (msec)</th>
<th>( V_{rev}^# ) (mV)</th>
<th>( G_{+80} / G_{-80}^@ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt GluR6</td>
<td>6.0 ± 0.2 (54)</td>
<td>1.9 ± 0.06 (35)</td>
<td>1.3 ± 1.3 (9)</td>
<td>1.5 ± 0.2 (14)</td>
</tr>
<tr>
<td>GluR6(S684C)</td>
<td>6.1 ± 0.1 (51)</td>
<td>2.0 ± 0.05 (29)</td>
<td>1.2 ± 1.4 (10)</td>
<td>1.8 ± 0.3 (10)</td>
</tr>
</tbody>
</table>

All measurements were made for current responses to rapid application of 1 mM glutamate for 100 msec.

*Desensitization time course was fit to a single exponential using CLAMPFIT software.

\(^\&\)Measured as time from 10 to 90% of peak current response.

\(^\#\)Holding potential was stepped from −60 mV to potentials ranging from −100 to +100 mV in 20 mV increments every 20 sec before application of glutamate; peak current responses were measured.

\(^@\)Ratio of conductance measured at +80 mV to that at −80 mV.

Data were collected and analyzed by Dr Raymond.
Figure 3. wt GluR6 shows significantly less cysteine-specific biotinylation than GluR6(S684C).

Representative Western blot analysis of wt GluR6 and GluR6(S684C) following incubation of transfected cells with NHS-SS-Biotin (primary amine-specific) or HPDP-Biotin (cysteine-specific), as described in Methods. 1, 2, 4, and 8% represent the fraction of the total cell membrane isolate, and “B” represents the total protein precipitated by streptavidin-linked beads, loaded in each lane.
Figure 4. Construction of standard curves for wt GluR6 and GluR6(S684C) biotinylated with NHS-SS-biotin and HPDP-biotin. For blots shown in figure 2, band intensities were measured by densitometry from lanes containing 1, 2, 4, and 8% of the total membrane protein in order to generate a standard curve. From these curves, the amount of biotinylated GluR6 was calculated as a fraction of the total membrane GluR6.
**Figure 5.** Bar graph representing the percentage of the total GluR6 receptors biotinylated and precipitated using streptavidin beads. Bars represent data from N=7-12 different experiments.

#, significant difference (p<0.05) between *wt* GluR6 and GluR6(S684C) for biotinylation by HPDP-Biotin; **, significant difference (p<0.001) for streptavidin bead recovery of *wt* GluR6 after incubation with NHS-SS-Biotin vs. HPDP-Biotin (both by unpaired *t* test).
Figure 6. Cys-specific biotinylation of GluR6(S684C) shows significant decrease in the presence of kainate. Representative Western blot analyses of wt GluR6 and GluR6(S684C) following incubation of transfected cells with HPDP - Biotin in the presence (+KA) vs. absence (-KA) of 1 mM kainate. Left and right panels are from same gel. Labeling of lanes is as described in figure 2.
Figure 7. Standard curve for wild type GluR6 and GluR6(S684C) in the presence vs. absence of kainate. The standard curves were generated from blots shown in figure 5, as described in figure 3. These curves were used to determine amount of Cys-biotinylated GluR6 as a fraction of the total membrane GluR6.
Figure 8. Ratio of Cys-biotinylated GluR6 in the presence vs. absence of kainate was calculated for each of N=8 (WT R6) or N=9 (R6(S684C)) different experiments. **, significant difference (p<0.01 by unpaired t test) between wt GluR6 and GluR6(S684C).
Figure 9. Occupation of ligand binding site and channel activation required to render Cys 684 inaccessible to HPDP-Biotin. Binding of another agonist, glutamate, decreases Cys-specific biotinylation of GluR6(S684C). Representative Western blot analysis of GluR6(S684C)-transfected cells treated with HPDP-Biotin in the absence vs. presence of 1 mM glutamate (Glu). Left and right panels are from same gel. Labeling of lanes is as described in figure 2.
**Figure 10.** Antagonist binding does not alter Cys-specific biotinylation of GluR6(S684C).

Representative Western blot analysis of GluR6(S684C)-transfected cells treated with HPDP-Biotin in the absence vs. presence of 10 μM CNQX. Left and right panels are from same gel. Labeling of lanes is as described in figure 2.
Figure 11. Standard curves were generated from blots shown in figures 8 and 9, as described in figure 3. Band intensities from 2, 4, and 8% of total membrane proteins were measured by densitometry to construct the curves. The curves were used to calculate the amount of biotinylated GluR6 as a percentage of the total membrane GluR6.
Figure 12. Ratio of Cys-biotinylated GluR6 in the presence vs. absence of Glu or CNQX was calculated from N=4 different experiments for each condition: WT R6, +Glu/-Glu; S684C, +Glu/-Glu; and S684C, +CNQX/-CNQX. *, significant difference (p<0.05 by unpaired t test) between wt GluR6 and GluR6(S684C) for +Glu/-Glu; #, significant difference (p<0.05, unpaired t test) between +Glu/-Glu and +CNQX/-CNQX for GluR6(S684C).
Figure 13. Cys-specific biotinylation decreases peak current amplitude and slows desensitization for both wt GluR6 and GluR6(S684C). Whole-cell patch clamp recordings were made under voltage clamp (V_h = -60 mV) from cells transfected with either wt GluR6 or GluR6(S684C). Upper panel: Representative current responses to rapid application of 1 mM glutamate (indicated by bar) before (“control”) and after (“HPDP”) a 15 minute incubation with 0.03 mg/ml extracellular HPDP-Biotin followed by a 6-8 minute washout period. Lower panel: The gain of each HPDP trace has been increased to match the peak current amplitude of the corresponding control trace to better illustrate the slowing of desensitization. Bars represent agonist application. Data were collected and analyzed by Dr Raymond.
Figure 14. Peak current amplitude following 15 minute incubation (and 6-8 minute washout) with 1% DMSO (vehicle), 0.03 mg/ml HPDP-Biotin (in 1% DMSO), or 0.03 mg/ml HPDP-Biotin in the continuous presence of 1 mM KA (+ KA), was normalized to the pretreatment peak current amplitude (I₀). Bars represent data from N = 5 different cells for vehicle (results from wt GluR6- and GluR6 (S684C)-transfected cells were pooled), N=11 for wt R6, N=9 for S684C, or N=8 for S684C + KA. **, significant difference between vehicle and HPDP-Biotin treated groups by unpaired t test, p<0.01. Data were collected and analyzed by Dr Raymond.
Figure 15. Desensitization time constant ($\tau_D$) following treatment with HPDP-Biotin (as in figure 13) was normalized to pretreatment value ($\tau_D$ (initial)). Bars represent data from $N = 5$ (vehicle), $N = 11$ (wt R6), $N = 9$ (S684C), and $N = 8$ (S684C + KA) different cells. Data were collected and analyzed by Dr Raymond.
**Figure 16.** Extracellular streptavidin slows desensitization of Cys-biotinylated GluR6(S684C).

Whole-cell patch clamp recordings were made from cells expressing *wt* GluR6 or mutant GluR6(S684C), as in figure12. Representative current responses to 1 mM glutamate recorded from three different cells following HPDP-Biotin treatment and wash-out period ("HPDP"; as in Fig. 12), and then 10-15 minute incubation with 0.01 mg/ml streptavidin followed by 6-8 minute wash-out ("SA"). In far right panel, cell was treated continuously with 1 mM kainate during the time of incubation with HPDP-Biotin ("HPDP + KA"). Bars represent agonist application. The data were collected and analyzed by Dr Raymond.
Figure 17. Peak current amplitude following streptavidin treatment and washout \( (I_{SA}) \) was normalized to peak current amplitude immediately following HPDP-Biotin treatment (with or without 1 mM kainate) and washout period \( (I_{HPDP+/-KA}) \). Bars represent data from \( N = 5 \) for the wt R6, -KA; \( N = 6 \) for S684C, -KA; and \( N = 5 \) for S684C, +1 mM KA. Data were collected and analyzed by Dr Raymond.
Figure 18. Desensitization time constant measured after incubation with streptavidin was normalized to that measured immediately prior to streptavidin treatment (as in figure 16). Bars represent data from N = 5 for wt R6, -KA; N=6 for S684C, -KA; or N=5 for S684C, +1 mM KA. *, significant difference between groups by unpaired t test, p=0.02. Data were collected and analyzed by Dr Raymond.
Figure 19. Sequence alignment of part of the M3-M4 loop of iGluRs.

- /// Indicates a stretch of ~21 amino acids.
- The amino acid numbering of cysteine residues (C, indicated in the parentheses) is based on the sequence of mature protein.
Table 2: Assessment of protein expression and function of GluR6 (wt and mutant) receptors.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Protein expression</th>
<th>Functional receptor</th>
<th>Glu EC\textsubscript{50} *</th>
<th>τD (ms)</th>
<th>HPDP-biotin precipitation</th>
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<tr>
<td>WT</td>
<td>Yes</td>
<td>Yes</td>
<td>~0.53</td>
<td>~5-7</td>
<td>+</td>
</tr>
<tr>
<td>C65S</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>~6-9 (n=3)</td>
<td>++</td>
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<tr>
<td>C199S</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>~8 (n=4)</td>
<td>+</td>
</tr>
<tr>
<td>C316S</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C432S</td>
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<td>C719S</td>
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<td>~16 (n=7)</td>
<td>++++</td>
</tr>
<tr>
<td>C773S</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C719S/C773S</td>
<td>No</td>
<td>No</td>
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ND = not determined
*EC\textsubscript{50} for glutamate.

** A faint band was observed.
Figure 20. GluR6(C719T) shows significantly more cysteine-specific biotinylation than wt GluR6. Representative Western blot analyses of wt GluR6 and GluR6(C719T) following incubation of transfected cells with HPDP-Biotin (cysteine-specific), as described in Methods. The numbers represent the amount of proteins in µg, and “B” represents the total protein precipitated by streptavidin-linked beads, loaded in each lane.
Figure 21. Ratio of change in Cys-biotinylated GluR6 (WT R6) and (R6(C719T)) from four different experiments (N=4). Ratio of change in biotinylation for C719T was obtained by comparing it to the WT R6 biotinylation. Significant increase in the amount of Cys-biotinylated protein was obtained for GluR6(C719T). **, significant difference (p<0.001 by unpaired t test) between wt GluR6 and GluR6(C719T).
Figure 22: Glutamate-induced desensitization in *wt* GluR6 and GluR6(C719T). Representative current traces recorded from cells expressing either *wt* GluR6 or GluR6(C719T). Agonist-dependent desensitization was prolonged in GluR6(C719T) expressing cells. The desensitization time constant, $\tau_D$, of glutamate-induced current was $15.7 \pm 1.1$, ~2.6 fold slower than that of *wt* GluR6. The mean 10-90% rise time was similar for both *wt* GluR6 and GluR6(C719T). The current calibration bar: *wt* GluR6 = 800 pA and GluR6(C719T) = 500 pA.
Figure 23. GluR6(C65S) shows significantly more cysteine-specific biotinylation than wt GluR6. Cysteine specific biotinylation of GluR6(C199S) is similar to wt GluR6. Representative Western blot analyses of wt GluR6 and GluR6(C199S) and GluR6(C65S) following incubation of transfected cells with HPDP-Biotin (cysteine-specific), as described in Methods. The numbers represent the amount of proteins in μg, and “B” represents the total protein precipitated by streptavidin-linked beads, loaded in each lane.
Figure 24. Ratio of change in Cys-biotinylated GluR6, (WT R6), (R6(C199S)) and (R6(C65S)) from four different experiments (N=4). The amount of WT R6 biotinylation was taken as 1 or 100% and the ratio of biotinylation for the mutant receptors were calculated accordingly. Significant increase in the amount of Cys-biotinylated protein was obtained for R6(C65S) but not for R6(C199S). **, significant difference (p<0.001 by unpaired t test) between wt GluR6 and R6(C65S).
DISCUSSION

SECTION I: Structure of M3-M4 loop.

We have used a combined biochemical and electrophysiological approach to analyze the structure of the M3-M4 loop using \textit{wt} GluR6 and GluR6(S684C) receptors. We examined the location of amino acid 684 in the presence or absence of an agonist following incubation with reagents that add biotin molecule(s) to cell surface proteins. Our data are consistent with an extracellular location for amino acid 684, both in the presence and absence of agonist. Our results also suggest that an agonist-induced conformational change in GluR6 reduces accessibility of this amino acid to the extracellular aqueous solution.

In our biochemical analyses of GluR6, we first determined the percentage of total membrane GluR6 receptors expressed at the cell surface using NHS-SS-Biotin. Since this reagent targets primary amines, every surface receptor should bind multiple biotin molecules and thus be isolated by streptavidin-linked bead precipitation. Moreover, the addition of one biotin molecule per receptor should be sufficient to recover the receptor by streptavidin-linked bead precipitation. Our data indicated that recovery of surface \textit{wt} GluR6 was reduced to \textasciitilde50\% when HPDP-Biotin was used as compared with NHS-SS-Biotin. On the other hand, the recovery of surface GluR6(S684C) was similar using either reagent. These results suggest that the cysteine residue substituted at position 684 within the M3-M4 loop is in the reduced state and freely accessible to extracellular reagents, whereas other reduced cysteine residues present in both \textit{wt} GluR6 and GluR6(S684C) are less accessible to these reagents.
There are, however, limitations in our biochemical analysis of biotinylated receptors. Our approach in showing that C684 is biotinylated was somewhat indirect. Other experiments may be carried out to show more directly that C684 in GluR6(S684C) is indeed biotinylated by extracellular Cys-specific reagents. In one approach, a non-cleaveable Cys-specific biotin, such as BMCC-biotin, can be used to biotinylate the surface receptors. The receptors will then be immunoprecipitated, and cleaved into fragments using specific proteases that cleave proteins at certain residues. The fragment containing the biotin molecule may then be detected using horse radish peroxidase (HRP)-conjugated streptavidin similar to the detection of the HRP-conjugated secondary antibody in Western blot analysis of GluR6 receptors. Alternatively, purified streptavidin may be added to the biotinylated fragment and the sample run on a gel. Since streptavidin is a large molecule (M, of ~60 KDa) the biotinylated fragment bound to streptavidin would run at a different speed on the gel and may be detected following staining of the gel. In fact, we have tried several times to detect the biotinylated fragments using HRP-conjugated streptavidin but probable loss of the fragments in the biochemical steps as well as a high background in Western blot of the biotinylated fragments prevented us from a successful detection of the fragments. Another alternative approach is to perform mass spectroscopy reading of the fragments after purifying the biotinylated fragment using an avidin column.

Several factors may affect the extent of Cys-specific biotinylation of wt and mutant GluR6 receptors including time, pH, and temperature. In our experiments, we have incubated the receptors with the biotin reagent for ~30 min. The fact that biotinylation of wt GluR6 using HPDP-biotin was ~50% of that using NHS-SS-biotin indicates that the reduced cysteine(s) in wt GluR6 might be within a globular protein environment not readily accessible to the extracellular biotinylating reagent. These data suggest that the biotinylation reaction may not go to completion
following a 30 min incubation with HPDP-biotin. On the other hand, longer incubation may not increase the extent of biotinylation further due to degradation of the HPDP-biotin over time at 37° C. Our second set of data (results, section II) on biotinylation of other GluR6 mutants (C719T, C65S, C199S) as well as wt GluR6 receptors indicated a low biotinylation and bead recovery of surface receptors (compare Fig. 3 with Fig 20 or 23 for wt GluR6). The biotinylation of these receptors was performed at 4° C (instead of 37° C) and in non-buffered medium (instead of a pH 7 medium), suggesting that temperature as well as pH may affect the extent of Cys-specific biotinylation.

For GluR6(S684C), two different results indicate that accessibility of Cys 684 to the Cys-specific biotinylating reagents, HPDP-Biotin, is significantly decreased in the presence of agonist: 1) the recovery of surface receptors by Cys-specific biotinylation and streptavidin-linked bead precipitation was reduced in the presence of agonist and 2) no effect on the channel function was observed after Cys-specific biotinylation followed by extracellular streptavidin in the presence of agonist. In addition, occupation of the ligand binding site does not seem to alter accessibility of Cys 684, as biotinylation of GluR6(S684C) was similar in the presence or absence of the competitive antagonist CNQX. Therefore, we conclude that an agonist-induced conformational change involved in channel activation and/or desensitization reduces accessibility of Cys 684. Since treatment of Cys-biotinylated GluR6(S684C) with extracellular streptavidin does not prevent channel activation, membrane translocation of Cys 684 is unlikely. It is more likely that channel activation or desensitization results in Cys 684 or a segment containing this residue becomes buried in the protein environment and thereby sheltered from the extracellular aqueous environment. These results may be generalized to position 684 of wt GluR6 since the channel characteristics of GluR6(S684C) receptors are essentially identical to wt GluR6.
receptors. Our results are in agreement with a recently proposed model in which position 684 is not exposed to the agonist binding cleft but is within a 32 amino acid segment which is flanked by two amino acids involved in agonist binding (Swanson et al. 1997). This region essentially lacks secondary structure, allowing full exposure to aqueous solution in the unliganded state. Analysis of three dimensional structures of a homologous protein, LAOBP, indicates large scale movement of rigid globular protein domains associated with ligand binding. Oh et al. (1993) have shown that the two lobes of the LAOBP that are connected by two segments are far apart in the unliganded structure, but these lobes (lobe I and lobe II) come in close contact with each other following ligand binding to the protein. They showed that movement of the lobe II is a consequence of rotation of the lobes about a hinge region. They proposed that ligand binding to the protein stabilizes the structure of the protein in a conformation in which the two lobes are in close contact. Assuming structural homology between LAOBP and GluRs, the lobe II would include a segment of the M3-M4 loop containing amino acid 684 of GluR6. It is possible that upon agonist binding, the amino acid residue 684 or a segment containing this residue is rotated from a position that is fully exposed to the extracellular aqueous environment to a position that is in close contact with protein and less exposed to the aqueous environment.

In our patch clamp recording experiments of GluR6, biotinylation of Cys residue(s) resulted in a decrease in peak current amplitude as well as slowing of desensitization. Since both mutant and wt GluR6 are similarly affected, the effect of biotinylation may be attributed to Cys-residues present in both mutant and wt GluR6 (i.e. not Cys 684). For wt GluR6 receptors subsequent treatment with streptavidin did not alter channel function further. The results of our biochemical analysis indicate that Cys-specific biotinylation of wt GluR6 receptors is incomplete, likely due to low accessibility of cysteine residue(s) to extracellular HPDP-Biotin.
Since streptavidin is a much larger molecule than HPDP-Biotin, perhaps its access to biotinylated cysteine(s) is extremely limited. In contrast, although biotinylation of Cys 684 appeared to be functionally silent, subsequent streptavidin treatment of GluR6(S684C) resulted in a further slowing of desensitization. Since other segments of the M3-M4 loop have been implicated in regulating desensitization of AMPA/KA receptors (Sommer et al., 1990; Lomeli et al., 1994; Mosbacher et al., 1994; Partin et al., 1996; Swanson et al., 1997), addition of a large molecule such as streptavidin at that site is likely to interfere with desensitization of the channel.

Our data are consistent with an entirely extracellular M3-M4 loop. The possibility of a dynamic structure for the M3-M4 loop was raised by Nakazawa et al. (1995). They showed that phosphorylation of Ser 696 in the AMPA receptor subunit GluR2 was agonist dependent. They suggested that Ser 696 of GluR2 may be exposed on the cytoplasmic side for phosphorylation by a conformational change after agonist binding to the receptor. Our data, however, do not support membrane translocation of Ser 684 and the surrounding regions upon agonist binding and channel gating. It is possible that membrane topology varies between subunits.

Moreover, Ser684 of GluR6 has been suggested as the PKA phosphorylation site and thus intracellular since mutation of this residue to Ala (S684A) significantly decreased or eliminated the potentiating effect of intracellular PKA. A number of possibilities may explain the effect of PKA phosphorylation observed in these studies as well as the phosphorylation studies by Nakazawa et al. (1995). One possibility is that PKA phosphorylation may occur at an extracellular site. This would be possible if phosphorylation of GluRs occurs while they are in the endoplasmic reticulum (ER). Moreover, it has been shown that protein kinases, including cAMP-dependent kinases, can act at the extracellular sites (Ehrlich et al. 1986; Kubler et al. 1992). It is also possible that PKA is released from the cell to phosphorylate an extracellular site.
Some cells such as platelets have been shown to release PKA upon stimulation (Korc-Grodzicki et al. 1988).

Another possibility is that phosphorylation may occur at a site on the C-terminus but Ser 684 is involved in relaying the phosphorylation signal to the channel. There are several potential PKA phosphorylation sites on the C-terminus of AMPA and kainate receptors. One such a site has been shown to be Ser 845 of AMPA receptor subunit GluR1 (Roche et al. 1996). It is possible that mutation of Ser 684 to Ala (S684A) in GluR6 alters the channel structure such that PKA phosphorylation of another residue on the C-terminus, for instance, is interrupted thereby eliminating the effect of PKA. Moreover, it has recently been shown that intracellular perfusion with activated PKA, during whole cell patch clamp recording of GluR6 receptors, increases the open probability of the channels by nearly 50%, from $P_{\text{open}}$ of ~0.6 to ~0.9 (Traynelis and Wahl, 1997). Ser 684 may be critical to channel gating and the Ser to Ala mutation may increase the $P_{\text{open}}$ of the channels thereby occluding the potentiating effect of intracellular PKA. In order to address these issues, one may examine the $P_{\text{open}}$ of the GluR6(S684A) receptors and determine whether intracellular PKA perfusion has any effect on the channel $P_{\text{open}}$. In addition, one could examine the potentiating effect of intracellular PKA perfusion in another Ser 684 mutant such as GluR6(S684C), and to determine if the $P_{\text{open}}$ of this mutant receptor differs from the wt GluR6 receptors.

In summary, the results of our experiments place the M3-M4 loop entirely extracellular. However, a dynamic M3-M4 loop structure in which upon agonist binding and channel gating putative phosphorylation sites, Ser 684 of GluR6, and the surrounding region translocate across the membrane is unlikely. The amino acid 684 within the M3-M4 loop appears to be involved in an agonist-induced conformational change associated with channel gating.
SECTION II: The role of cysteine residues.

In our analysis of the role of Cys-residues in the structure and function of GluR6 receptors, we first mutated putative extracellular Cys-residues to Ser or Thr and tested if the mutant receptors were expressed on the cell surface and could form functional channels. Patch clamp recordings of cells transfected with the mutant receptors C719S, C719T, C199S, and C65S indicate that these receptors form functional channels suggesting proper folding and insertion of the receptors into the membrane.

The ligand-binding domain of ionotropic glutamate receptors is attributed to two discontinuous regions of the protein, S1 and S2 segments, which form a bilobed agonist-binding pocket. Moreover, residues within the S2 segment have been shown to affect desensitization of AMPA/KA receptors. For GluR6(C719T), the slowing of desensitization onset for glutamate-induced currents might be due to loss of a disulfide bond formed by Cys719. This may result in disruption of tight coupling of the structures involved in channel activation and desensitization. Moreover, if Cys719 in GluR6 is involved in a disulfide bond (i.e. oxidized form) mutagenic removal of this residue would free the second Cys-residue (i.e. reduced form) rendering it available for Cys-specific biotinylation.

Our biochemical analysis of GluR6 receptors indicates a significant increase in Cys-specific biotinylation of the mutant GluR6(C719T) suggesting that Cys719 is involved in a disulfide bond and that its Cys-residue partner is freely accessible to the extracellular environment. A residue that possibly forms disulfide bond with Cys 719 is Cys 773. Previous
studies on the role of Cys-residues in the modulation of GluRs indicated that homologous Cys-
residues in NMDA receptors form disulfide bond (Sullivan et al. 1994). Moreover, analysis of
ligand binding domain of GluRs indicated existence of a disulfide bond formed by homologous
Cys-residues in AMPA receptors (Abele et al. 1998; Armstrong et al. 1998). However, there is
no direct evidence for the existence of this disulfide bond between these two residues in intact
AMPA/kainate receptors.

In our hands, mutation of Cys773 to Ser (C773S) as well as a double mutation
(Cys719S/C773S) resulted in loss of protein expression in HEK-293 cells suggesting that
Cys773 is important in the stability and expression of the protein. It is possible that a disulfide
formed by Cys773 is critical to the stability of the protein and that removing this bond would
result in loss of protein expression or in degradation of the protein by intracellular enzymes. One
possibility is that in GluR6, Cys719 may not form a disulfide bond with Cys773 but with another
Cys-residue within N-terminal region of the protein. Moreover, mutation of Cys316 to Ser
(C316S) also resulted in loss of protein expression. It is possible that Cys316 forms a disulfide
bond with Cys773 and that reducing this bond by mutagenic removal of either Cys 316 or Cys
773 may result in structural instability of the protein. Alternatively, the loss of protein expression
(i.e. protein instability) of both C773S and C316S mutants might be a result of the serine
mutation. To test this possibility, further mutation should be made, substituting other amino
acids for Cys-residue at positions 773 and 316.

Mutation of Cys 432 to Ser (C432S) also resulted in an unstable protein. However, the
protein expression of this mutant seems to be somewhat different than C773S and C316S
mutations. A faint band was observed in the Western blot analysis of the C432S receptor. It was
not possible to assess biotinylation of C432S receptors due to low protein expression. Moreover,
it is not clear whether the rate of translation of the protein is low for this mutant or if the proteins
are degraded more quickly. Further mutation of Cys 432 residue to amino acids other than serine
may address this issue.

Biochemical analysis of GluR6(C65S) also indicates increased Cys-specific biotinylation
of this mutant receptor. Using high-resolution crystallography, it has been shown that cysteine
residues in a bacterial periplasmic binding protein, leucine/isoleucine/valine-binding protein
(LIVBP), form a disulfide bond (Sack et al. 1989). The LIVBP has high sequence homology
with the N-terminal region of iGluRs (O’Hara et al. 1993). The disulfide bond-forming Cys-
residues in the LIVBP are homologous to Cys 65 and Cys 91 in GluR6. It is possible that these
two residues form a disulfide bond in GluR6 receptors. The role of these residues and first ~400
amino acids of the N-terminal region in the structure and function of AMPA/KA receptors are
yet to be determined. Further experiments are required to determine which Cys-residue forms a
disulfide bond with Cys 65 and Cys 719 and to assess the role of these residues in the structure
and function of GluR6 receptors.

In summary, our data indicates that Cys 65 and Cys 719 are involved in disulfide bonds
with other Cys-residues in GluR6. Cys 199 does not appear to form disulfide bond in the wt
receptor and is likely to be in the reduced state. Alternatively, its Cys partner may be inaccessible
to extracellular HPDP-biotin. Cys 719 is involved in desensitization of the channel perhaps
through a disulfide-bond that holds structures involved in agonist binding and desensitization
together. Cys 773 and Cys 316 appear to be critical in the stability and expression of the
receptors. Our data suggest that Cys 719 may not form a disulfide bond with Cys 773 but with
other cysteine residues within N-terminus region of GluR6 receptors.
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