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ACCELERATED COMMUNICATION

Identification of a Site in GluR1 and GluR2 That Is Important for Modulation of Deactivation and Desensitization

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ABSTRACT

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid subtype of ionotropic glutamate receptors consists of rapidly gating ion channels. Positive modulation of channel gating may slow gating kinetics through at least two distinct mechanisms, evidenced by the predominant slowing of either the rate of receptor desensitization or the rate of offset after agonist withdrawal (deactivation). This study compares the actions of two positive allosteric modulators [cyclothiazide, which modulates desensitization, and 1-(1,4-benzodioxan-6-ylcarbonyl)piperidine (CX546), which modulates deactivation] in a mutant shown previously to impede modulation by cyclothiazide. These experiments test the hypothesis that the point mutation, GluR1(S493T), would also cause a loss of modulation by CX546. Wild-type GluR1 through -4 receptors were modulated by CX546, as assayed by the potentiation of steady-state currents in the *Xenopus laevis* oocyte expression system. CX546 potentiated steady-state currents of both splice isoforms of GluR1. Modulation by CX546 was completely abolished in GluR1(S493T) and its homolog, GluR2(S497T), although this mutation did not affect apparent agonist affinity in the absence of CX546. Thus, the GluR1(S493T) mutation has a similar impairment of modulation by either cyclothiazide or CX546, indicating that some residues at the subunit interface of glutamate receptors play an important role in channel deactivation and desensitization.

Ionotropic glutamate receptors are responsible for fast excitatory synaptic transmission in the central nervous system (Dingledine et al., 1999). Subtypes of glutamate receptors contribute to neuroplasticity: in animal models of learning and memory, *N*-methyl-D-aspartate receptors induce neuroplasticity, whereas the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of ionotropic glutamate receptors maintain the potentiated state (Huang and Stevens, 1998). In some cases, the potentiated state is maintained through cellular mechanisms that increase both the number and activity of AMPA receptors (Isaac et al., 1999). Thus, both *N*-methyl-D-aspartate and AMPA receptors may be important targets for the development of pharmacological agents that improve cognitive function and thereby treat brain damage or disease (Lynch, 1998).

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Two known classes of drugs act on AMPA receptors and have cognition-enhancing actions: the benzamides, including the AMPAkine CX546 and aniracetam; and the benzothiadiazides (BTDs), including cyclothiazide (CTZ) and IDRA21 (Isaacson and Nicoll, 1991; Yamada and Rothman, 1992; Zivkovic et al., 1995; Arai et al., 2002a). The AMPAkines have shown promise in improving cognitive function in laboratory animals and humans (Granger et al., 1993; Staubli et al., 1994; Arai et al., 1996b; Ingvar et al., 1997; Lynch et al., 1997). In particular, there are several recent reports on the mechanism of action of the AMPAkines CX516, CX546, and CX614 (Arai et al., 1996a; Arai et al., 2000, 2002b; Nagarajan et al., 2001). A potential problem for therapeutic use of cognition-enhancing drugs is that excessive AMPA receptor activity may induce hyperexcitability and seizures (Pelletier and Hablitz, 1996; Yamada, 1998). Thus, the development of brain-region–specific and subtype-specific drugs is an important goal that may require understanding the molecular mechanisms of these drugs.

Cognition-enhancing drugs modulate AMPA receptor gat-

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CTZ, cyclothiazide; CX546, 1-(1,4-benzodioxan-6-ylcarbonyl)piperidine; BTD, benzothiadiazide; GluR, ionotropic glutamate receptor; IDRA21, 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; WT, wild type.

ing, including channel desensitization and deactivation; they also may modulate agonist affinity. Desensitization is the process by which the channel moves to a nonconducting state, although the agonist remains bound, thereby uncoupling ligand binding from channel opening. In contrast, deactivation is channel closure in the absence of desensitization; experimentally, this is measured as the offset of current after the withdrawal of the agonist (τ_{deact}). Thus, measurements of deactivation do not resolve channel closure from agonist unbinding. Generally, drugs such as benzamides are believed to modulate AMPA receptors by slowing deactivation. However, it has been proposed that there are different subfamilies of benzamides having distinct modulatory properties (Arai et al., 2002b). These investigators proposed that CX546 is a member of the subfamily that primarily slows channel closing rates (increasing τ_{deact}); this delays entry into the desensitized state, resulting in a secondary slowing of the macroscopic rate of channel desensitization (increasing τ_{des}). Alternatively, Nagarajan et al. (2001) suggested that CX546 slows agonist unbinding and increases the rate out of the desensitized state. Their hypothesis was supported by their findings that a nondesensitizing mutant receptor was still modulated by CX546, by virtue of the increase in agonist affinity. Either of these actions of benzamides is in contrast to the proposed actions of BTDs, including CTZ, which slow or block entry into the desensitized state with only a modest secondary effect on channel deactivation (Partin et al., 1996).

Consistent with the conventional idea that allosteric transitions involve intersubunit interactions, Sun et al. (2002) proposed that movement of AMPA receptor subunits at a dimer interface mediates desensitization. Using the isolated GluR2 agonist-binding domain, they found that CTZ spans this interface by forming hydrogen bonds and nonpolar contacts with amino acids from both subunits. CTZ may stabilize the interface and subsequently block transition of the receptor into the desensitized state. Thus, CTZ stabilizes the ligand-bound, nondesensitized conformation of AMPA receptors.

We previously mutated a residue at the subunit interface, GluR1(S493T), that impedes CTZ modulation of desensitization (Partin, 2001). These results are supported by structural data, which show that the homologous residue in GluR2, Ser497, hydrogen bonds with CTZ (Sun et al., 2002). Taken together, these data further implicate the subunit interface in desensitization and BTD action. In contrast, the binding site(s) of modulators of deactivation (e.g., CX546) have not been characterized. Modulator binding is presumed to be extracellular and coupled allosterically to the agonist-binding domain because the effects of CX546 depend on agonist concentration (Nagarajan et al., 2001).

The present study tests the hypothesis that the mutation of GluR1(S493T) also alters modulation by CX546. This assumes that channel deactivation (channel closing) requires a concerted conformational change at a dimer interface. To test this hypothesis, we compared CX546 and CTZ modulation on mutant and wild-type AMPA receptors. The results indicate that GluR1 Ser493 mutations severely impair modulation by CX546 as well as by CTZ, suggesting that a common binding site exists.

Materials and Methods

Plasmids and Mutants. Plasmids encoding cDNAs for flip (i) and flop (o) variants of wild-type GluR1 through -4 were gifts of Dr. Peter Seeburg (University of Heidelberg, Heidelberg, Germany) and Dr. Stephen Heinemann (Salk Institute, La Jolla, CA). Site-directed mutagenesis (QuikChange Mutagenesis Kit; Stratagene, La Jolla, CA) was performed to generate GluR1(S493T) and GluR2(S497T) mutations, which were confirmed by DNA sequencing (Macromolecular Resources Facility, Colorado State University; Fort Collins, CO).

Oocyte Expression. Capped mRNA was synthesized in vitro from linearized AMPA receptor cDNAs using T3 (GluR3) or T7 (GluR1, -2, and -4) polymerase (mMessage Machine; Ambion, Austin, TX). Oocytes were surgically obtained from adult *Xenopus laevis* (Nasco, Fort Atkinson, WI) anesthetized by immersion in 3% Tricaine (Sigma, St. Louis, MO) for 15 min and then placed on an ice bed. Animal care and surgical procedures conformed to institutional Animal Care and Use Committee standards and practices and were carried out in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Harvested ovarian lobes were cut into small pieces and incubated with 1.5 mg/ml collagenase A (Roche Diagnostics, Indianapolis, IN) in calcium-free buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) for 90 min at room temperature on a Nutator (Clay Adams, Parsippany, NJ). After thorough washing [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES, pH 7.6], selected eggs were stored at 18°C and injected 16 to 24 h later. RNA (46 nl) at 0.5 to 1.0 μg/μl was injected into the oocyte cytoplasm with the use of a Drummond positive-displacement injector using micropipettes pulled to a diameter of $<$10 μm. To increase the channel conductance of homooligomeric WT GluR2, RNA was made from the pore mutant GluR2(R607Q) (the same mutation also referred to as "$GluR2_Q$" by Nagarajan et al., 2001). The receptors made from this mutant conducted robust current and had an inwardly rectifying current-voltage relationship. GluR2(S497T) was constructed within this pore mutant background and therefore is a double mutant.

Oocyte Electrophysiology. Experiments on oocytes were performed under two-electrode voltage clamp (Axoclamp 2A or GeneClamp 500B; Axon Instruments, Union City, CA) at a holding potential of −60 mV in a continuously perfused chamber of approximately 5 μl volume. The extracellular solution was calcium-free [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ba(NO_3)_2$, 0.41 mM $BaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES, pH 7.6], to which was added glutamate, cyclothiazide (20 mM stock solution dissolved in DMSO), or CX546 (40 mM stock solution dissolved in DMSO) (all drugs were purchased from Sigma/RBI (Natick, MA). Drug concentrations used to determine the EC_{50} values in Table 1 were: CTZ, 1–300 μM; CX546, 1–1500 μM; and glutamate, 0.1–10,000 μM. Solution exchange was controlled via an electronic BPS-8 valve control system (ALA Scientific, Westbury, NY) and electronic valves (The Lee Co., Westbrook, CT). Drugs were applied for 60 s. Currents were measured at steady-state levels at the end of each application of drug. Electrodes of 0.1 to 3 MΩ resistance were filled with 1 M CsCl and 5 mM EGTA. Current responses were filtered at 100 Hz (Cygnus Technology, Delaware Water Gap, PA) and acquired by a Power Macintosh 7600/132 computer with an ITC-16 (InstruTECH Corporation, Port Washington, NY) interface under control of the program Synapse (Synergistic Research Systems, Silver Spring, MD).

Oocyte Data Analysis. Data were analyzed and graphed using KaleidaGraph (Abelbeck/Synergy Software, Reading, PA). Dose-response analysis was with either glutamate or CX546, as appropriate. The lack of solubility of CX546 limited the ability to test its effects beyond 1500 μM. Responses were fitted to the logistic equation $I = I_{max} \times (1/(1 + (EC_{50}/[ligand])^{nH}))$, where I_{max} is the response at a saturating concentration of ligand, EC_{50} is the concentration of ligand producing a half-maximal response, and n_H is the Hill coefficient. Statistical analysis of the significance of differences between groups (single-factor analysis of variance) was performed using Microsoft Excel software (Microsoft, Redmond, WA).

Transient Transfections. Human embryonic kidney (HEK) 293 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured as described previously (Partin et al., 1996). Briefly, cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gemini, Irvine, CA), penicillin/streptomycin (100 units/ml each), and 1% GlutaMax-1 (both from Invitrogen, Carlsbad, CA). Cells were transiently transfected using FuGene 6 reagent (Roche Diagnostics) with GluR2o cDNA and enhanced green fluorescent protein cDNA (0.5 and 0.1 μg/35-mm dish, respectively).

Outside-Out Patch Recordings. Currents were recorded 48 to 96 h after transfection. During recordings, cells were perfused with extracellular solution containing 20 mM sucrose, 145 mM NaCl, 5.4 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 1.8 mM $CaCl_2{\cdot}H_2O$, and 0.01 mg/ml phenol red, pH 7.3. Outside-out membrane patches from transfected HEK293 cells were voltage-clamped at a holding potential of −60 mV using an Axopatch 200B amplifier (Axon Instruments). Synapse software (ver. 3.6d; Synergistic Research Systems) was used to control piezoelectric movement, data acquisition, and trace analysis. Responses were filtered at 5 kHz, digitized at 10 to 500 μs/point, and stored on a Power Macintosh computer using an ITC-16 interface (InstruTECH). Thin-walled borosilicate glass micropipettes (World Precision Instruments, New Haven, CT) with a resistance of 2 to 5 MΩ were filled with 135 mM CsCl, 10 mM CsF, 10 mM HEPES, 5 mM Cs-BAPTA, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$, pH 7.2, 292 mOsm. Patches were perfused with a θ tube (Sutter Instrument Co., Novato, CA) flowpipe using two pairs of solutions. The first pair assessed baseline responses to glutamate. The vehicle-control barrel contained 145 mM NaCl, 5.4 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 1.8 mM $CaCl_2{\cdot}2H_2O$, DMSO (0.3–1%), and 0.01 mg/ml phenol red, pH 7.3; the glutamate-containing barrel included glutamate (10 mM) in addition to the above components. The pair of solutions used for testing CX546 were as above, but with CX546 (300 μM) added to both the control (CX546-control) and glutamate-containing (CX546 + glutamate) barrels. A higher concentration of CX546 (1 mM) was used in several pilot studies, but a 300 μM concentration was used for subsequent experiments because of problems with response run-down, patch stability, and concerns about the high DMSO concentrations. CX546 stock (100 mM) was dissolved in DMSO. The flowpipe solutions were driven continuously by a syringe pump (KD Scientific, New Hope, PA) at 0.3 ml/min. After going into voltage clamp, an outside-out patch was pulled and then lifted up to the flowpipe. The pipette tip was positioned in the stream containing control extracellular solution near the interface between the glutamate-free and glutamate-containing solutions. To assess the response to glutamate alone, the patch pipette was jumped rapidly from the vehicle-control into the glutamate solution; for assessing the drug effect, the pipette was jumped from CX546-control into CX546 + glutamate. Rapid solution exchanges of 1 or 100 ms were driven by a piezoelectric device (Burleigh Instruments, Fishers, NY). Solution exchange rates were determined at the end of each experiment by open-tip junction currents. The average 10 to 90% rise times of the 1-ms pulses were 0.29 to 0.32 ms per experimental group (range, 0.19 to 0.44 ms). Data were excluded with rise times of >0.5 ms.

Analysis of Rapid Responses. The rate of deactivation was estimated by fitting a single exponential (τ_{deact}) to responses evoked by a 1-ms pulse of glutamate. The rate of desensitization was estimated by fitting the decay of the response to a 100-ms glutamate pulse from 95% of peak to the steady state with a single-exponential function (τ_{des}). There was substantial desensitization during the initial component of responses to 1-ms glutamate pulses. Accordingly, the rate of deactivation was estimated from fits to the subsequent fast decay after the end of the glutamate pulse. In all cases, deactivation was faster than desensitization, such that there was a clear increase in the rate of decay after removal of glutamate. For all responses, deactivation kinetics were measured from that point out to 4 to 6 ms from the peak amplitude. This measurement accounted for 100% of the current decay in all patches, except for responses in WT patches treated with CX546. In those patches, the measurements accounted for 72 to 98% of the current decay, so the majority of the decay was included in the fit; points beyond this cutoff were disrupted by a solution artifact in some experiments. Current traces and graphs were plotted using KaleidaGraph 3.5 (Abelbeck/Synergy). Patch responses are averages of 3 to 20 successive glutamate applications.

Results and Discussion

Effects of the S493T Point Mutation on Modulation by CTZ and CX546. The GluR1i(S493T) mutation was shown previously to impair the modulation of desensitization by 100 μM CTZ (Partin, 2001), which is consistent with the fact that the homologous residue in GluR2, Ser497, lies at the subunit interface and interacts with CTZ (Sun et al., 2002). Figure 1 demonstrates that the GluR1(S493T) mutation impairs modulation by CX546 (Fig. 1A) and CTZ (Fig. 1B) in both splice isoforms, as assayed by slow perfusion in *X. laevis* oocytes.

CTZ had a higher apparent affinity for GluR1i (EC_{50} = 58 μM) versus GluR1o (EC_{50} = 237 μM) for potentiating glutamate-evoked responses (Table 1), as has been demonstrated previously using kainate as the agonist (Partin et al., 1994). As expected, CTZ was also more efficacious at GluR1i, potentiating glutamate-evoked responses with 300 μM CTZ 40-fold, whereas GluR1o responses were potentiated only 8-fold. The Ser493

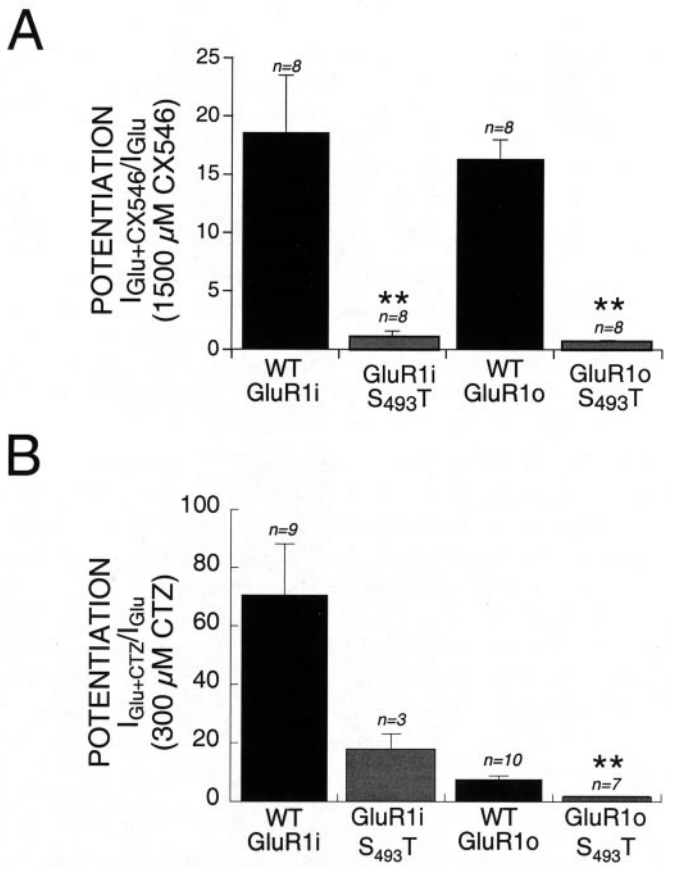

Fig. 1. The S493T mutation impedes the modulation of desensitization and deactivation in flip and flop AMPA receptor isoforms. A, mean potentiation of steady-state current evoked by 300 μM glutamate with 1500 μM CX546 for GluR1i and GluR1o wild-type (■) and the Ser493 mutant (▨), expressed in oocytes. For both splice isoforms, this mutation significantly blocks modulation by CX546 ($p < 0.05$). B, mean potentiation by CTZ. For both splice isoforms, this mutation also impedes the action of CTZ.

TABLE 1

Loss of modulation of GluR1(S493T) by CTZ and CX546

Loss of modulation by both CX546 and CTZ occurs when mutations are made at either position 493 (this article) or position 750 (this article and Partin et al., 1995) in GluR1. Values represent mean ± S.E.M.; n = 5 to 14 oocytes, but most data points represent n = 8. Hill values are given in parentheses below the value for EC_{50}.

	CTZ		CX546		Glutamate
	EC_{50}	Potentiation (300 μM CTZ) $I_{GLU + CTZ}/I_{GLU}$	EC_{50}	Potentiation (1500 μM CX546) $I_{GLU + CX546}/I_{GLU}$	EC_{50}
	μM		μM		μM
WT GluR1i	58.0 ± 1.9 (2.2)	39.6 ± 7.2	503 ± 73 (1.6)	18.5 ± 4.9	27.1 ± 8.9
GluR1i(S493T)	N.D.	17.9 ± 5.2	N.D.	1.1 ± 0.5	21.9 ± 7.3
WT GluR1o	237 ± 104 (0.9)	8.7 ± 1.7	595 ± 315 (1.0)	16.3 ± 1.7	11.1 ± 7.4
GluR1o(S493T)	N.D.	1.8 ± 0.1	N.D.	0.7 ± 0.1	28.3 ± 11.8
GluR1o(S750Q)	N.D.	1.0 ± 0.1[a]	N.D.	1.8 ± 0.2	N.D.

N.D., not determined (i.e., data could not be fit reliably with logistic equation because of small current amplitudes).
[a] Data from Partin et al. (1995).

mutation had a similar effect on both GluR1i and GluR1o receptors, reducing the level of maximal potentiation to 18- and 2-fold, respectively (Fig. 1B). These data indicate that mutation of Ser493 in either the flip or flop isoform of GluR1 has a deleterious effect on allosteric modulation by CTZ. This finding is consistent with the structural data for the S1/S2 domain complexed with both agonist and CTZ (Sun et al., 2002).

To test the hypothesis that the mutation affecting CTZ modulation would also affect CX546 modulation, similar analyses on WT and mutant GluR1i and GluR1o receptors were performed (Table 1). There was no difference in apparent affinity of CX546 for GluR1o (EC_{50} = 595 μM) versus GluR1i (EC_{50} = 503 μM), and the responses were potentiated to a similar extent (Fig. 1A). For both splice isoforms, the Ser493 mutation completely abolished potentiation by CX546. Thus, the Ser493 point mutation impaired modulation by both CX546 and CTZ, although these two drugs are believed to act through distinct mechanisms.

Previous experiments demonstrated that the AMPA receptor alternatively spliced flip/flop region was responsible for the differential sensitivity to CTZ and aniracetam (Partin et al., 1996). The differential sensitivity could be mapped to one critical residue, Ser750 (or Asn750 in the flop isoform) and, in fact, modulation could be abolished with the mutation GluR1(S750Q). Because CX546 is structurally related to aniracetam, one might predict that modulation by CX546 would be impaired by mutation of Ser750. Indeed, Table 1 shows that modulation of GluR1o(S750Q) by CX546 is reduced to 1.8-fold. Thus, there is a significant loss of modulation by both CX546 and CTZ upon mutation of both Ser493 and Ser750.

The loss of modulation by the Ser493 mutation could be a secondary consequence of a reduction in agonist affinity. To test this idea, glutamate dose-response analyses were performed on WT and mutant GluR1i and GluR1o receptors. The Ser493 mutation did not significantly change the apparent affinity of glutamate (Table 1). The EC_{50} for WT GluR1i was 27 μM and 22 μM for GluR1i(S493T); for WT GluR1o, the EC_{50} was 11 μM and 28 μM for the mutation. In addition, the mutation did not significantly change the efficacy of glutamate, because the mean peak current for WT GluR1i in 1000 μM glutamate was 414 ± 60 nA (n = 20) versus 569 ± 72 nA (n = 16) for GluR1i(S493T) (p = 0.10). Thus, the Ser493 mutation impairs modulation by CTZ and CX546, similar to the effects of the GluR1o(S750Q) mutation (Table 1), which impairs modulation by CTZ and aniracetam (Partin et al., 1996), without affecting inherent agonist affinity.

TABLE 2

Modulation of AMPA subunits by CX546

CX546 modulates the glutamate-evoked currents of the flop (o) isoform of all AMPA subunits. Values reported are the fit of the data from dose-response analysis using 1 to 1500 μM CX546, with n = 8 oocytes per determination. The left column shows the mean EC_{50}; given the limited solubility of CX546, these values may represent a lower estimate of the actual value (see also Nagarajan et al., 2001). Right column shows the mean potentiation of the current evoked by 300 μM glutamate and 1500 μM CX546.

	EC_{50} CX546	1500 μM CX546 $I_{GLU + CX546}/I_{GLU}$
	μM	
GluR1o	595 ± 315 (1.0)	24.7 ± 5.5
GluR2o (R607Q)	176 ± 26 (1.6)	9.9 ± 0.5
GluR3o	554 ± 159 (2.4)	30.9 ± 5.3
GluR4o	681 ± 566 (1.3)	8.8 ± 3.5

Modulation of All AMPA Receptor Subtypes by CX546. A dose-response analysis was used to test the efficacy of CX546 modulation on different AMPA receptor subtypes (Table 2). For these experiments, the flop splice isoform was used. Responses from GluR1 to -4 receptors were potentiated by CX546. There were 2- to 3-fold differences in the apparent affinities (EC_{50} values ranged from 176 to 681 μM) and 3-fold differences in the efficacy (maximal potentiation ranged from 10- to 31-fold) of CX546. Thus, CX546 positively modulates the flop isoform of all AMPA receptors. To interpret the electrophysiological experiments in light of the published crystal structural data of GluR2, we next performed kinetic experiments using GluR2o.

Kinetic Analysis of GluR2o(S497T). Nagarajan et al. (2001) have shown previously that CX546 modulation of "$GluR2i_Q$" caused a small but significant increase of the time constant of deactivation. In Fig. 2A, a similar result is shown for GluR2o, as assayed in outside-out patches of transiently transfected HEK293 cells, using ultrafast solution perfusion. CX546 slowed deactivation (i.e., increased τ_{deact}) from 0.71 ± 0.05 to 1.10 ± 0.08 ms (p = 0.001) (Fig. $2C_1$) without significantly changing τ_{des} (Fig. $2C_2$). In addition, CX546 significantly increased the ratio of steady-state to peak current from 0.06 ± 0.03 to 0.43 ± 0.04 ($p < 0.001$) (Fig. 2, A_2 and C_3). This modulation most likely arises as an indirect effect of CX546, which alters equilibrium desensitization by slowing channel deactivation rather than directly affecting the rate of desensitization (Partin et al., 1996; Arai et al., 2002b). The fact that CX546 did not alter τ_{des} may reflect that our experiments were performed in subsaturating concentrations of CX546 (300 μM). When we performed pilot experiments at a higher concentration of CX546 (1000 μM), the steady-state/peak ratio increased to near unity (S. Clark and K. M. Partin, unpublished observations).

The homolog of GluR1(S493T) is GluR2(S497T). Kinetic analysis of GluR2o(S497T) is shown in Fig. 2B. The ability of CX546 to modulate deactivation is completely lost in GluR2o(S497T). The loss of modulation also was evident as a decrease in equilibrium desensitization, as measured by the change in $I_{ss/peak}$ (Fig. 2C_3).

Identification of a Site Important for the Modulation of Deactivation and Desensitization. The data shown above indicate that mutation of Ser493 in GluR1 or Ser497 in GluR2 impairs AMPA receptor modulation by either class of drug. Fig. 3 shows the crystal structure of a dimer of the S1/S2 agonist-binding domain of GluR2o with glutamate bound (Armstrong et al., 1999). Ser497 lies at the dimer interface that has been shown to participate in CTZ binding. Mutation from serine to threonine impairs modulation by CTZ. The mutation also disrupts modulation by CX546, suggesting either that CX546 also binds at this site or that mutation of this residue perturbs the receptor structure and/or function in such a manner as to make it insensitive to allosteric drugs.

Summary. Oligomeric ligand-binding proteins rely on subunit interface interactions for the regulation of allosteric transitions (Creighton, 1993). For ionotropic glutamate re-

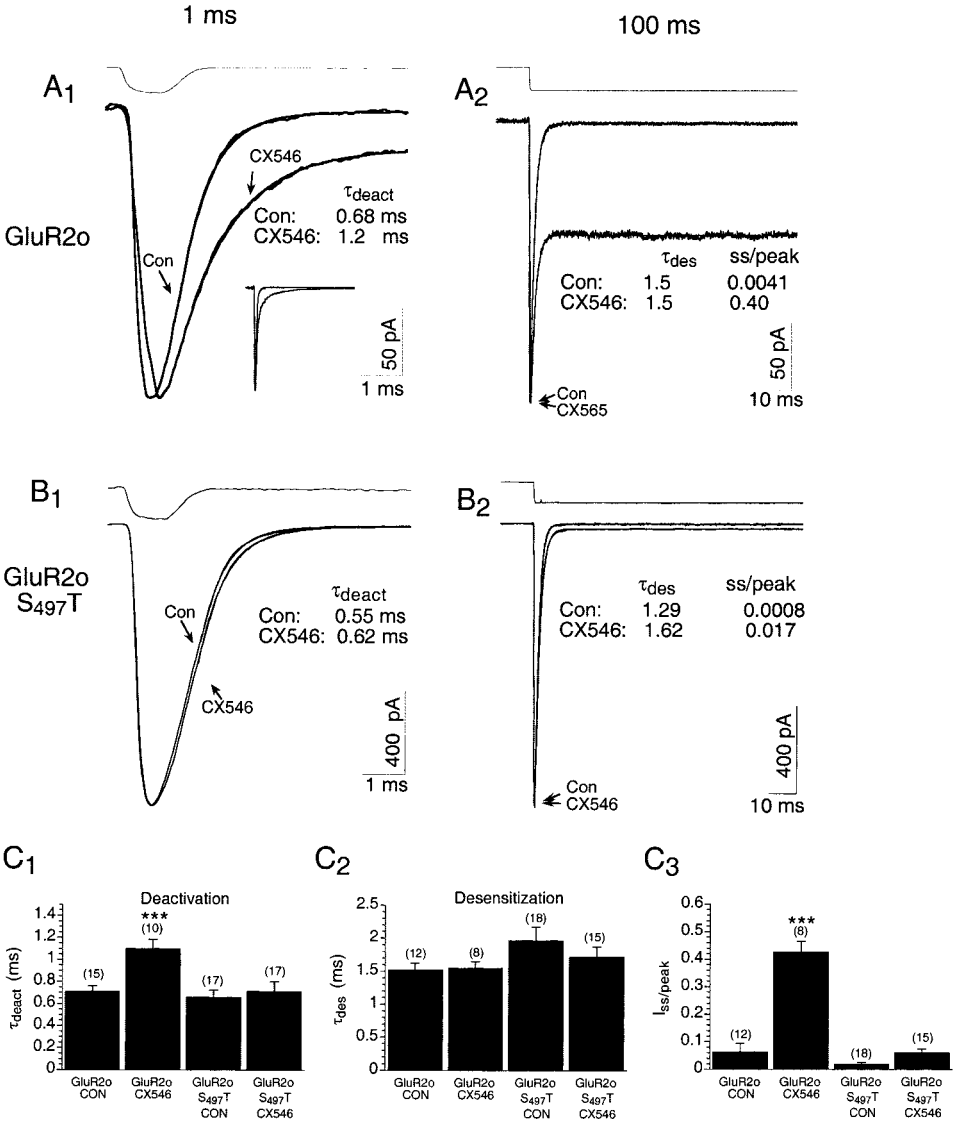

Fig. 2. Kinetic analysis of GluR2oand GluR2o(S497T). A, responses in outside-out patches of HEK293 cells expressing GluR2o, with a 1-ms (A_1) or 100-ms (A_2) pulses of glutamate (10 mM) (Con) or glutamate (10 mM) + CX546 (300 μM) (CX546). Inset traces in A_1 show that the decay in CX546 returns to baseline within 70 ms. CX546 slowed deactivation (τ_{deact}), increased $I_{ss/peak}$, but did not alter τ_{des}. All four traces are from the same patch. B, responses in GluR2o(S497T) patches. As shown, CX546 no longer modulated deactivation (B_1), nor potentiated the steady-state/peak current ratio (B_2). All four traces are from the same GluR2o(S497T) patch. Fits for individual waveforms are displayed; arrows indicate the single-exponential fits for analysis of deactivation or desensitization. For each pair of traces, inset values show time constants for deactivation and desensitization, as well as the steady-state/peak current ratios for each trace. Traces are normalized to peak amplitudes to correct for rundown. For each pair of traces, the amplitude of the smaller response (and fit) was scaled to the larger. (Accordingly, the calibration bars apply only to unscaled traces.) Scaling factors are as follows: GluR2o, 1 ms (1.047) and 100 ms (1.61); and GluR2o(S497T) 1 ms (1.055) and 100 ms (1.060). Shown above the current traces for A and B are open-tip junction currents. C, bar graphs for deactivation (C_1), desensitization (C_2), and steady-state/peak current ratios (C_3). Data given are group means (± S.E.M.); values in parentheses indicate the number of patches analyzed. C_1: ***, CX546 significantly slowed deactivation in GluR2o ($p \leq 0.001$). In contrast, GluR2o(S497T) abolished modulation of deactivation by CX546. C_2, desensitization rates were not affected by CX546. C_3, the modulatory effect of CX546 with respect to $I_{ss/peak}$ was greatly attenuated by the GluR2o(S497T) point mutation. ***, CX546 significantly increased the ss/peak ratio in GluR2o ($p \leq 0.001$). GluR2o refers to GluR2o(R607Q), as discussed under *Materials and Methods*.

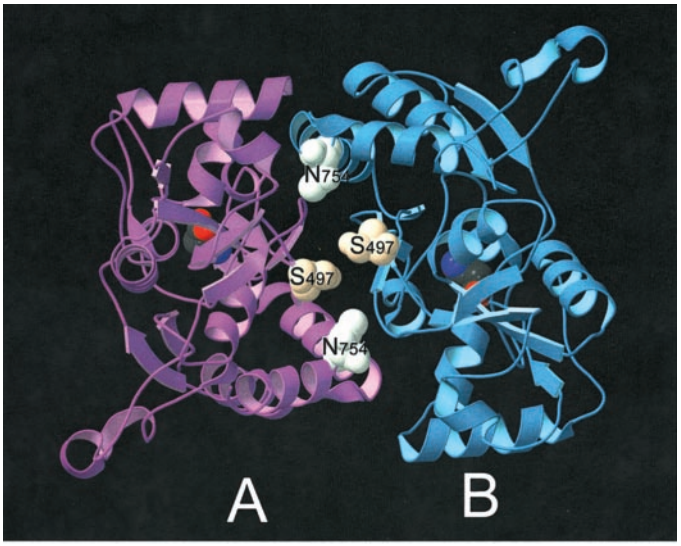

Fig. 3. Structure of the GluR2o S1/S2 agonist-binding domain, with glutamate bound. Ser497 lies at the interface between subunits A and B, as does Asn754 [homologous to GluR1o(Asn750) within the flip/flop splice region (Armstrong and Gouaux, 1999; structure is PDB file "1FTJ")]. Mutation of either residue impairs modulation by either CTZ or CX546, suggesting a common or overlapping binding site.

ceptors, such transitions after ligand binding include channel activation, deactivation, and desensitization. It is assumed that these transitions are coupled (Partin et al., 1996; Trussell and Otis, 1996), although this process is poorly understood. We have shown that one residue, GluR1(S493T) or GluR2(S497T), plays a critical role in permitting allosteric modulation of these gating transitions, presumably by participating directly in the drug-protein interaction. The role of this residue in regulating allosteric modulation seems to be similar to that of GluR1(Ser750) or GluR2(Ser754), the mutation of which also results in a receptor that is poorly modulated by either CTZ or aniracetam (Partin et al., 1995, 1996). Both of these residues (Ser493 and Ser750) directly participate in the binding of CTZ (Sun et al., 2002). The present studies suggest that modulation by CX546 may also be directed by these two residues. However, mutation of GluR1(S493T) had a more profound impact on modulation by CX546 than by CTZ (Table 1). This could suggest either that there are fewer contacts between CX546 and the receptor (so each contact is relatively more significant) or perhaps that Ser493 is a uniquely important contact for modulation of deactivation. In summary, mutations at the subunit interface of glutamate receptors impede the actions of drugs that modulate both deactivation and desensitization. It remains to be seen whether all such residues also participate in gating transitions and the extent to which they are involved in coupling these distinct allosteric processes.

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