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Effects of the Potent Ampakine CX614 on Hippocampal and Recombinant AMPA Receptors: Interactions with Cyclothiazide and GYKI 52466

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ABSTRACT

R,*S*-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor up-modulators of the benzamide type ("ampakines") have previously been shown to enhance excitatory synaptic transmission in vivo and in vitro and AMPA receptor currents in excised patches. The present study analyzed the effects of an ampakine (CX614; 2*H*,3*H*,6a*H*-pyrrolidino[2″,1″-3′,2′]1,3-oxazino[6′,5′-5,4]benzo[*e*]1,4-dioxan-10-one) that belongs to a benzoxazine subgroup characterized by greater structural rigidity and higher potency. CX614 enhanced the size (amplitude and duration) of field excitatory postsynaptic potentials in hippocampal slices and autaptically evoked excitatory postsynaptic currents in neuronal cultures with EC_{50} values of 20 to 40 μM. The compound blocked desensitization (EC_{50} = 44 μM) and slowed deactivation of responses to glutamate by a factor of 8.4 in excised patches. Currents through homomeric, recombinant AMPA receptors were enhanced with EC_{50} values that did not differ greatly across GluR1–3 flop subunits (19–37 μM) but revealed slightly lower potency at corresponding flip variants. Competition experiments using modulation of [^{3}H]fluorowillardiine binding suggested that CX614 and cyclothiazide share a common binding site but cyclothiazide seems to bind to an additional site not recognized by the ampakine. CX614 did not reverse the effect of GYKI 52466 on responses to brief glutamate pulses, which indicates that they act through separate sites, a conclusion that was confirmed in binding experiments. In sum, these results extend prior evidence that ampakines are effective in enhancing synaptic responses, most likely by slowing deactivation, and that their effects are exerted through sites that are only in part shared with other modulators.

The list of compounds that modulate *R*,*S*-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors has grown steadily since the nootropic drug aniracetam was discovered to increase currents mediated by the receptors (Ito et al., 1990). Although aniracetam proved useful for experimental purposes (Staubli et al., 1990; Isaacson and Nicoll, 1991; Tang et al., 1991), it has been largely supplanted by more potent and metabolically stable compounds. The first of these to be introduced were diazoxide and cyclothiazide (CTZ), two benzothiadiazides used clinically as antihypertensives or diuretics. CTZ potently blocks the AMPA receptor's rapid desensitization (Yamada and Rothman, 1992; Yamada and Tang, 1993). Surprisingly, given their potent effects in excised patch studies, the benzothiadiazides proved to have very modest effects on the size and wave form of synaptic responses in hippocampal slices (Randle et al., 1993; Larson et al., 1994; Arai and Lynch, 1998b). Re-examination in patches showed that although CTZ is indeed potent in blocking desensitization during long agonist applications, it produces only modest changes when very brief pulses, corresponding to transmitter release events, were used (Arai and Lynch, 1998a,b). These observations led to the conclusion that desensitization, at least in the hippocampus, plays a minor role in shaping synaptic responses. Recent work using isolated synaptic responses has confirmed this conclusion (Hjelmstad et al., 1999).

Ampakines constitute a second, still evolving group of modulators that were originally derived from aniracetam. Unlike the benzothiadiazides, ampakines cause rapid, fully reversible, and pronounced increases in hippocampal synaptic responses (Arai et al., 1994; Staubli et al., 1994a,b) without detectably affecting membrane potential or inhibitory transmission (Arai et al., 1996b). Effects on desensitization in excised patches vary with drug structure but in most cases are less pronounced than those seen with CTZ. However,

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ABBREVIATIONS: AMPA, *R*,*S*-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CTZ, cyclothiazide; GYKI, GYKI 52466; EPSP, excitatory postsynaptic potential; DMSO, dimethyl sulfoxide; MEM, minimal essential medium; AP5, 2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; EPSC, excitatory postsynaptic current.

ampakines are considerably more effective in prolonging patch responses to 1-ms pulses of glutamate (Arai et al., 1996b; Arai and Lynch, 1998a). These results have led to the conclusions that ampakines slow both deactivation (channel closing, transmitter dissociation) and desensitization rates and that they modify transmission because of the former effect (Arai and Lynch, 1998b).

The different although overlapping effects produced by these two drug groups suggest the possibility that they recognize different sites on the AMPA receptor and/or have different affinities for AMPA receptor subunits. With regard to the latter question, experiments using recombinant, homomeric receptors established that aniracetam (Partin et al., 1996) does not have the marked flip/flop preferences exhibited by CTZ, and binding tests suggested a similar conclusion for ampakines (Hennegriff et al., 1997; Kessler et al., 1998). Tentative evidence for distinct sites on the receptor has been provided by the observation that aniracetam and CTZ respond differently to point mutations (Partin et al., 1996). However, most studies on ampakines were carried out with drugs that had much lower apparent affinity than CTZ. This difference in affinity could by itself be a decisive factor in that perhaps only receptor-drug interactions involving a minimal amount of binding energy might be able to produce the particular effects of a compound such as CTZ.

Continuing progress in resolving structure-activity relationships has now resulted in ampakines with at least 10-fold higher potencies. The early generation drugs contained two separate ring structures connected through a carbonyl group ("BDP" in Fig. 1). In one subgroup of ampakines, referred to as benzoxazines, these two elements have been connected via a heteroatom, which thus closes an additional ring and confers greater rigidity. The benzoxazine CX614 (2*H*,3*H*,6a*H*-pyrrolidino[2″,1″-3′,2′]1,3-oxazino[6′,5′-5,4]benzo[*e*]1,4-dioxan-10-one; Fig. 1) was used in the present study to compare its effects with those described previously for CTZ with regard to synaptic currents, aspects of receptor kinetics (such as deactivation and desensitization), subunit preferences, and agonist binding, and to test for competitive interactions with CTZ. Drug interactions were also studied between CX614 and GYKI 52466 (GYKI, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride; Tarnawa et al., 1992), a member of a third group of modulators that *reduce* AMPA receptor currents. Initial suggestions that GYKI compounds are inverse modulators of the CTZ site (e.g., Zorumski et al., 1993) were not confirmed by later analyses (e.g., Johansen et al., 1995; Kessler et al., 1996; Partin and Mayer, 1996; Yamada and Turetsky, 1996), but the possibility of a similar relationship with ampakines remained to be explored because GYKI shares a benzodioxole element in its structure with some of the latter drugs.

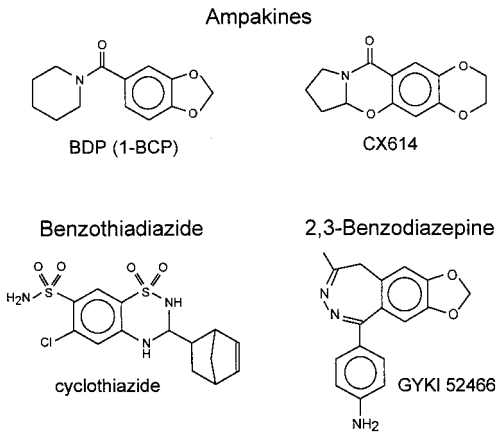

Fig. 1. Chemical structure of AMPA receptor modulators

Experimental Procedures

Extracellular Recording in Hippocampal Slices. Preparation of slices and recording methods have been described previously (Arai et al., 1996b). In brief, 400-μm slices were prepared from male Sprague-Dawley rats (150–200 g) that had been decapitated under anesthesia. The slices were transferred to a linear interface chamber perfused with oxygenated artificial cerebrospinal fluid (0.5 ml/min, 35°C). Field excitatory postsynaptic potentials (EPSPs) were recorded from the stratum radiatum with stimulation intensity adjusted to provide 50% of the maximum EPSP amplitude. Drug containing medium was prepared from a 500 mM stock solution of CX614 in dimethyl sulfoxide (DMSO) and was infused into the recording chamber with a syringe pump; the highest DMSO concentration of 0.2% did not influence synaptic transmission.

Whole-Cell Recordings from Hippocampal Primary Cultures. Whole-cell recordings were made from neuronal cultures prepared from the hippocampus with a slight modification of the method of Baughman et al. (1991). In brief, hippocampi from E16–18 Sprague-Dawley rats were isolated in ice-cold minimal essential medium (MEM) and cut into small pieces. The tissue was incubated with 0.05% trypsin/0.53 mM EDTA at 37°C for 30 min. After centrifugation (900 rpm), the tissue pellet was suspended in plating MEM containing 5% fetal calf serum, penicillin/streptomycin, 10 μM MK-801, and 100 μM 2-amino-5-phosphonopentanoic acid (AP5), and was gently triturated with Pasteur pipettes of various tip diameters until the cells were completely dispersed. The cell suspension was plated onto a recording chamber (Nunc, Naperville, CT) with microislands coated with (poly)D-lysine (0.02 mg/ml). The cells were fed every week by replacing approximately one-third of the medium containing 5% fetal calf serum (without NMDA receptor antagonists) and were grown for 10 to 25 days at 37°C. Whole-cell recordings were made from islands containing a solitary neuron (for autaptic response) or multiple cells (for asynchronous activity) with mature morphological characteristics (i.e., elaborate dendritic arbors with primary and higher order branches). The extracellular recording solution contained 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, and 20 mM HEPES, pH 7.37, and was supplemented with 50 μM picrotoxin, 10 μM MK-801, and 100 μM AP5. The intrapipette solution contained 130 mM CsF, 10 mM EGTA, 2 mM ATP disodium salt, and 10 mM HEPES, pH 7.4. Holding potential was −60 mV or as indicated. Both autaptic and asynchronously induced responses were evoked by clamping the membrane potential at +20 mV for 1 ms. Neurons from which recordings were made were identified immunohistochemically by fixing with 4% paraformaldehyde and visualizing with antibodies against MAP2, neurofilament, or synaptophysin.

Excised-Patch Recordings. Patch clamp studies were carried out with outside-out patches excised from CA1 pyramidal neurons of organotypic hippocampal slices. Slice cultures were prepared from 13 to 14-day-old Sprague-Dawley rats and grown for 2 weeks on cellulose membrane inserts (Millipore CM; Millipore Corporation, Bedford, MA) in an incubator (Arai et al., 1996b). For recording, a slice was transferred to a chamber and immersed in a medium containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 25 mM D-glucose, and 20 mM HEPES, pH 7.3. A patch was excised and relocated to an adjacent recording chamber perfused with recording medium containing: 130 mM NaCl, 3.5 mM KCl, 20 mM HEPES, 0.01 mM MK-801, and 0.05 mM D-AP5. Patch pipettes had a resistance of 3 to 8 MΩ and were filled with a

solution containing 65 mM CsF, 65 mM CsCl, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP disodium salt, and 10 mM HEPES, pH 7.3.

A piezo system (50-μm translation in 0.4 ms) was employed to rapidly switch solutions applied to the patch (Arai et al., 1996a,b; see also Colquhoun et al., 1992). In brief, background medium and glutamate-containing medium were flowing continuously through two lines of a double-barrel pipette with a θ-shaped cross-section. Both flow lines could be switched between multiple reservoirs. The excised patch was initially positioned in the background stream. Actuation of the piezo translator moved the double barrel pipette such that the patch was exposed to the glutamate flow line, and then returned to the original position after a predetermined time of 1 ms or longer. Typically, five responses were collected and averaged. For tests involving drugs, both background and glutamate flow lines were switched to solutions containing the drug of interest at the same concentration, and after about 30 s of equilibration, testing with glutamate pulses was resumed. Measurements with a given patch were alternated repeatedly between control condition and various test conditions with drug. For data analysis, responses with drug were compared with the averaged control responses taken before and after the drug response. Holding potentials were −50 mV. Data were acquired with a patch amplifier (AxoPatch-1D) at a filter frequency of 5 kHz and digitized at 10 kHz with PClamp/Digidata 1200 (Axon Instruments, Burlingame, CA). Deactivation rates were determined by fitting the decay phase of 1-ms pulse with a single-exponential or, if necessary, two-exponential function. CX614 solutions were prepared from a 500 mM stock solution in DMSO; the highest DMSO concentrations were 0.2% in the dose-response study and 0.5% when measuring competition with GYKI. The same final concentration of DMSO was included in all drug and control solutions.

Whole-Cell Recordings from HEK 293 Cells. Patch-clamp recordings were carried out in whole-cell configuration. HEK 293 cells that stably express homomeric AMPA receptors (rat GluR1–3; Yamada and Turetsky, 1996; Hennegriff et al., 1997) were transferred to a recording chamber (Nunc) at least a day before the experiment. Recordings were made at 25°C in serum-free MEM. Patch pipettes (3–7 MΩ) were filled with a solution containing 130 mM CsF, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM disodium ATP salt, and 10 mM HEPES, pH 7.4. The holding potential was −100 mV. Agonist application was made with a fast-solution switch system in which cells are exposed to a constant flow of the background solution that is momentarily stopped on application of the glutamate-containing medium. The drugs were included in both background- and agonist-containing solutions. Recordings with each cell were alternated between reference measurements (glutamate + 300 μM CX614) and test conditions. Data analysis was carried out as described above and concentration-response relations for CX614 and CTZ were constructed relative to the response obtained with 300 μM CX614.

Binding Assays. Rat brain membranes were prepared from the telencephalon according to conventional procedures (Kessler et al., 1996), which involved differential centrifugation to obtain a P_2 pellet fraction, osmotic lysis and repeated washing by centrifugation, and resuspending in the assay buffer (100 mM HEPES/Tris, 50 μM EGTA, pH 7.4). Frozen aliquots (−80°C) were thawed, sonicated, and washed twice by centrifugation. For tests with recombinant receptors, HEK 293 cells were suspended into physiological saline, collected by low-speed centrifugation (1,000*g* for 5 min), and resuspended in neutral 10 mM Tris/acetate. The cells were then homogenized by tip sonication and spun down at 45,000*g* for 30 min. The last step was repeated, after which the membranes were suspended in the assay buffer of 50 mM HEPES/Tris, pH 7.4. Binding tests with rat brain membranes were conducted at 25°C with the centrifugation method. Aliquots of the membrane suspension were incubated with radiolabeled compound and appropriate additions. Sets of 24 samples were then centrifuged for 20 min at 25,000*g* with rotor temperature maintained at 25°C. The supernatant was aspirated and the pellet quickly rinsed with ice-cold saline plus 50 mM potassium thiocyanate (wash buffer). Binding incubations with HEK 293 cell membranes were carried out at 0°C and terminated by filtration through GF/C filters after dilution in 5 ml of ice-cold wash buffer; the filters were rapidly washed with 3 volumes of additional wash buffer. Drugs were added from 100-fold concentrated solutions in DMSO; separate mixing tests verified that these procedures do not result in drug precipitation. Control samples received the equivalent amount of DMSO (maximum, 2%). Background values ("nonspecific binding") were measured by inclusion of 5 mM L-glutamate and subtracted from total binding; separate background values were determined for incubations with and without drug. Protein content was determined according to Bradford (1976) with the reagent available from Bio-Rad and with bovine serum albumin as standard. Binding curves were fitted to the data points through nonlinear regression (Prism; GraphPad, San Diego, CA).

Materials. [^{3}H]6-Cyano-7-nitro-quinoxaline-2,3-dione (CNQX) was purchased from NEN/DuPont (Boston, MA), [^{3}H]fluorowillardiine from Tocris Cookson (St. Louis, MO). Chemicals to prepare physiological media were from Sigma (St. Louis, MO). MEM was obtained from Life Technologies (Gaithersburg, MD). Cyclothiazide, GYKI, MK-801, and AP5 were purchased from RBI (Natick, MA). Nunc recording chambers for cell culture are distributed by Fisher (Pittsburgh, PA). Sprague-Dawley rats were obtained from Charles River (Wilmington, MA); the animals were housed, cared for, and sacrificed according to an institutionally approved protocol and the guidelines established by the National Institutes of Health.

Results

Effect of CX614 on Field EPSPs and Excitatory Postsynaptic Currents (EPSCs). Effects of CX614 on field EPSPs were examined in stratum radiatum of hippocampal field CA1 in slices that were maintained at 35°C in an interface chamber and continuously subfused with oxygenated artificial cerebrospinal fluid. Application of 30 μM CX614 produced an increase of ~40% in the amplitude and ~80% in the half-width of the response. This effect had a rapid onset, peaked within 20 min of drug application, and was fully reversed after 1 h of washing out the drug (Fig. 2, A and B). Significant changes in EPSP amplitude and half-width were observed at concentrations as low as 5 μM (Fig. 2A, right). Dose-response relations plotted in Fig. 2C provide EC_{50} estimates on the order of 30 μM for the amplitude and 18 μM (n_H = 1.7) for the half-width of the response; maximal effects could not be reliably determined because concentrations of 100 μM and above usually caused instability.

Drug effects on EPSCs were measured in cultured neurons grown on poly(lysine) microislands; only cells exhibiting mature morphological characteristics of pyramidal neurons were used. All recordings were carried out in the presence of 50 μM picrotoxin, 10 μM MK-801, and 100 μM AP5. In general, autaptic responses were obtained if microislands contained a solitary neuron. In these cases, a brief depolarizing pulse of 1 to 2 ms triggered a rapid inward current through voltage-dependent conductances (not shown), followed by a delayed inward current with a peak amplitude between 0.3 and 10 nA that decayed to baseline with a time constant of 4 to 13 ms (Fig. 3, A and B). The later current was completely abolished by application of 20 μM CNQX (Fig. 3A). The onset of the delayed inward currents varied, presumably because of differences in the length of axons and the location of active synapses. CX614 at 100 μM increased the autaptic EPSCs, the most reliable effect being a prolongation of the decay phase by a factor of 1.7 ± 0.1 (9.9 ± 2.5 ms for CX614 versus 5.5 ± 1.2 ms for predrug, seven experiments;

Fig. 3B). The increase in the amplitude was smaller (1.3 ± 0.2 times) and less reliable.

If microislands contained multiple interconnected neurons, a stimulation pulse delivered to one of them often triggered asynchronous synaptic responses, as shown in Fig. 3C. Twenty to eighty such events were analyzed per experiment. These responses in most cases had amplitudes of 20 to 60 pA and decayed to baseline with time constants of 2 to 12 ms. CX614 did not change the number of events per minute (17.4 ± 10.3 for drug versus 18.5 ± 9.4 for control, $n = 4$) but increased the responses, mainly by prolonging the decay time constant (from 7.4 ± 2.5 ms to 20.6 ± 5.4 ms; or by a factor of 3.0 ± 0.7, $n = 4$) with only a modest effect on the amplitude (1.1 times) (Fig. 3, D and E).

Effect of CX614 on AMPA Receptors in Excised Patches: Comparison with CTZ. Drug effects on AMPA receptor kinetics were analyzed in patches excised from CA1 pyramidal cells of cultured hippocampal slices in the presence of AP5 and MK-801. Step application of 1 mM glutamate produced an inward current that declined because of receptor desensitization to a steady-state level of 5 to 10% with a time constant of about 10 ms (Fig. 4; Arai et al., 1995, 1996a,b). CX614 raised the steady-state current without affecting the rate at which it was reached and it abolished any decay of the response at concentrations above 300 μM. The response profile at intermediate drug concentrations probably represents a superposition of the current produced by one subpopulation of receptors that have bound CX614 and do not desensitize plus the current from the remaining receptors that have not bound the drug. The steady-state current thus provides a measure for the fraction of receptors that have bound the drug. A dose-response relation is shown on the left side of Fig. 4 in which the steady-state current is expressed as a percentage of the peak response without drug. The EC_{50} value was 43 μM (n_H = 1.08) and the maximum was about 200%. Glutamate at 1 mM produces about half-saturation (Arai et al., 1995); thus, responses at high concentrations of CX614 reached about the same amplitude as responses produced by saturating concentrations of glutamate alone. This accords with previous observations using other modulators (Arai et al., 1996a) and with the finding that the EC_{50} for glutamate in general is shifted toward lower concentrations by such

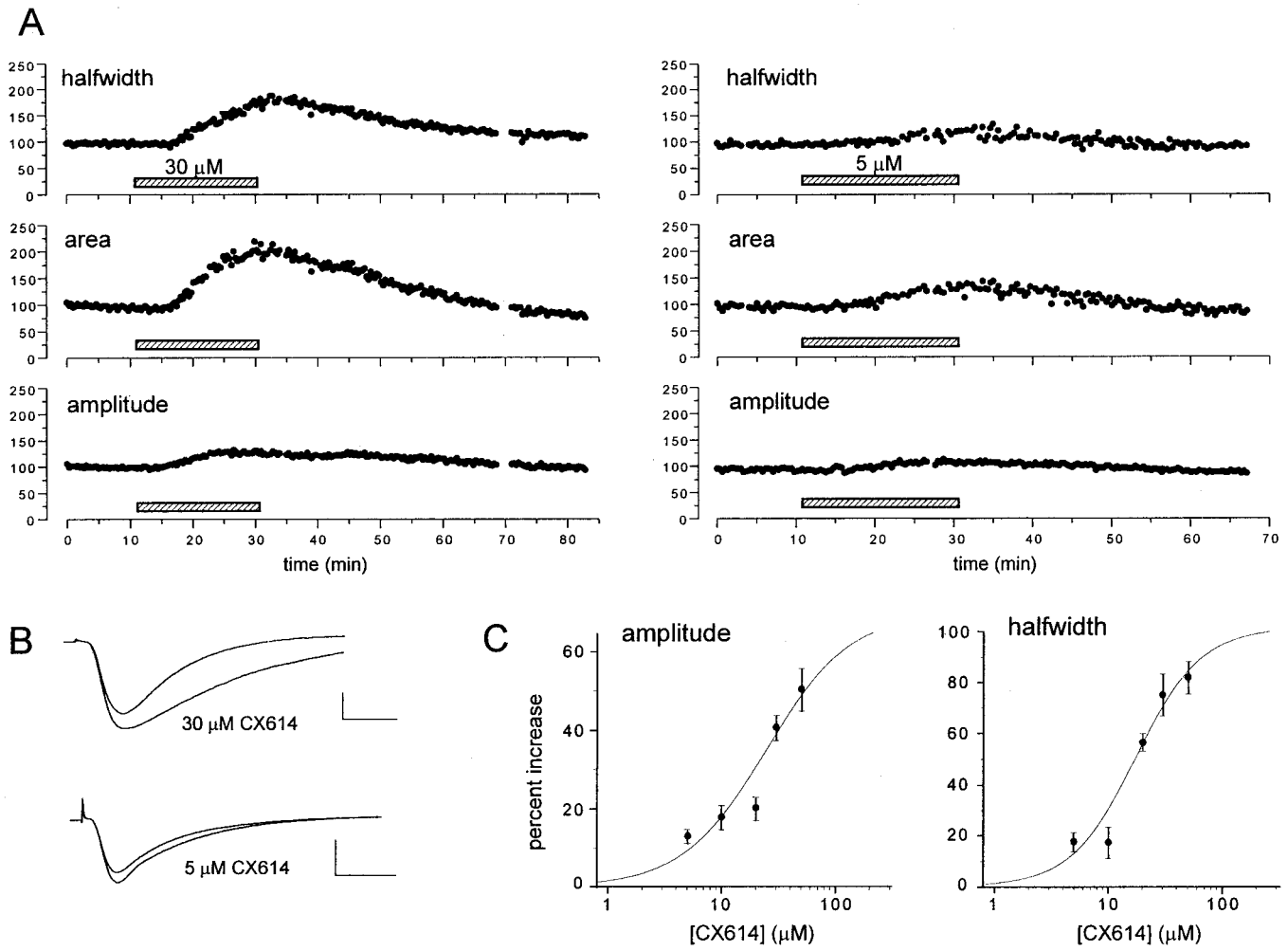

Fig. 2. Effect of CX614 on synaptic transmission in hippocampal field CA1. Recording and stimulation electrodes were placed in stratum radiatum of the field CA1 of a hippocampal slice. After establishing a stable baseline, CX614 (5–50 μM) was introduced into the perfusion line. Half-width and amplitude of the EPSP and area under the EPSP curve were normalized to those of the baseline responses and plotted against time. A, representative experiments with 30 μM (left) and 5 μM CX614 (right). The horizontal bar indicates the time of drug infusion. B, traces taken from the experiments shown in A. Calibration, 1 mV/10 ms. C, summarized data (mean and S.E.M.) of four experiments at each drug concentration. For EC_{50} estimates, see text and Table 1.

drugs (Yamada and Tang, 1993). The shift in the EC_{50} value of the peak current toward higher concentration (Fig. 4B) probably reflects a delay in the time to peak at subsaturating drug concentrations.

Figure 5 offers a more extensive kinetic analysis that includes the effects on responses induced by 1-ms applications of glutamate and a comparison with CTZ. These experiments were carried out with a saturating, 10-mM concentration of glutamate. Under these conditions, both drugs at 100 μM almost completely blocked the decay of the response induced by long (800 ms) application of glutamate [i.e., they increased the ratio between steady-state and peak current to 96 $\pm$ 2% (CX614; 10 patches) and 93 $\pm$ 3% (CTZ; 8 patches) (Fig. 5A)]. A substantial difference was seen, however, when glutamate was applied for only 1 ms. Control responses induced with this paradigm reached a peak after 0.5 ms; then, on removal of the agonist, they returned to baseline with a time constant of 2.6 $\pm$ 0.3 ms (n = 8). This deactivation phase of the response was greatly prolonged in the presence of 100 μM CX614. The decay phase seemed to contain a fast and a slow

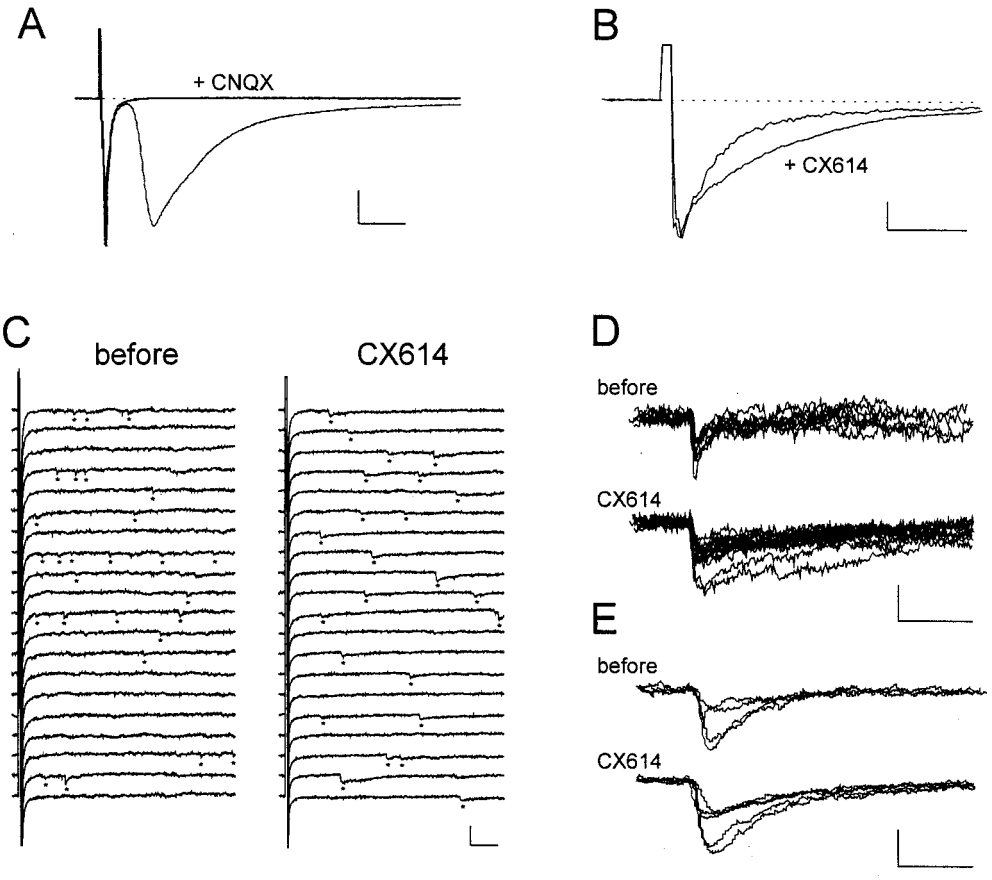

Fig. 3. Effect of CX614 on autaptic responses and asynchronous EPSCs in hippocampal neurons grown on microislands. A, superposition of autaptic responses obtained before and during application of 20 μM CNQX; representative experiment. Autaptic responses were evoked by delivering a brief depolarizing pulse through the recording electrode. The decay time constant of the response is 12.6 ms. Calibration, 2.5 nA/10 ms. B, autaptic responses before and after infusion of 100 μM CX614. Decay time constants are 4.4 and 10 ms, respectively. Calibration, 250 pA/10 ms. C, Effect of 100 μM CX614 on asynchronous synaptic activity triggered by depolarization pulses. Calibration, 200 pA/50 ms. D and E, superposition of asynchronous EPSCs recorded before and after infusion of CX614. Traces in D were taken from the experiment shown in C, as marked by asterisks; those in E are from a separate experiment that exhibited very large responses. Calibration for D, 50 pA/25 ms; for E, 200 pA/25 ms.

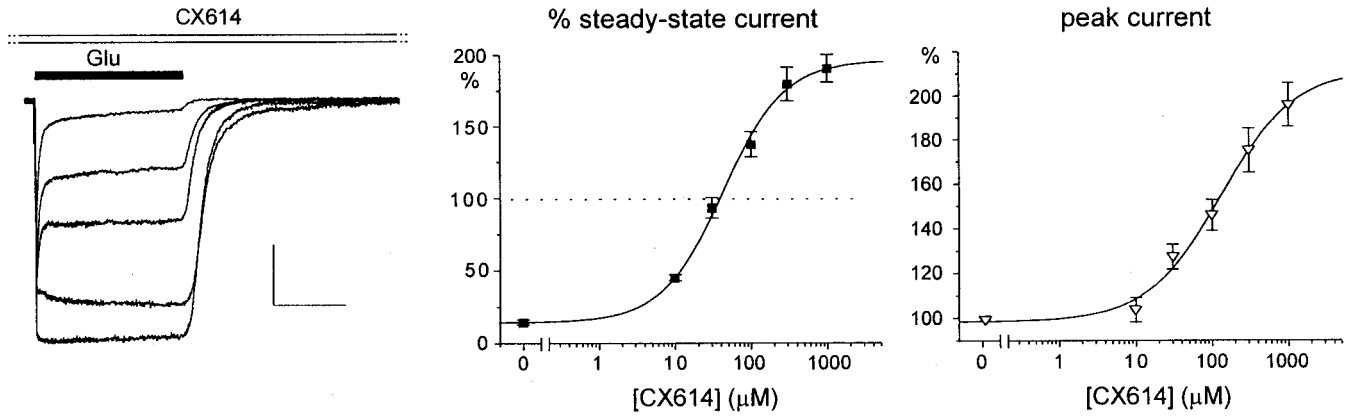

Fig. 4. Effect of CX614 on glutamate-induced currents in patches excised from CA1 pyramidal cells. Left, representative set of responses to 1 mM glutamate at increasing concentrations of CX614, recorded from a single patch. The patch was pre-equilibrated with drug before glutamate application. Holding potential, −50 mV. Calibration, 100 pA/200 ms. Right, steady-state and peak currents were normalized to those induced by 1 mM glutamate alone, and percentage changes over control responses were plotted on the y-axis against drug concentration. Each point (mean and S.E.M.) represents data averaged from seven to nine patches. EC_{50} values for the steady-state current and the peak current were 43.7 μM (n_H = 0.76) and 132 μM (n_H = 0.9), respectively.

component, presumably because the concentration of 100 μM did not saturate the receptors; the time constants for these two components were 3.7 ± 0.7 ms (54 ± 4% of amplitude) and 20.5 ± 3.6 ms (46 ± 4%). The slow component was increased by a factor of 8.4 ± 1.5 over the control value (six pairs). The effect of CTZ by comparison was moderate (4.2 ± 0.6 ms versus 2.9 ± 0.2 ms, six pairs).

The bottom row of Fig. 5 shows a second approach to assess receptor desensitization. Paired application of 1-ms glutamate pulses typically results in a reduction in the amplitude of the second response by about half, presumably because some receptors activated during the first glutamate application converted to the desensitized state (Colquhoun et al., 1992; Arai and Lynch, 1996). At high concentrations, both drugs completely suppressed this paired-pulse reduction, but CX614 was slightly less potent, in that its effect was incomplete at 100 μM (Fig. 5C, left and middle). Taken together, the results of Figs. 4 and 5 show that CX614 effectively blocks desensitization and that it is similar in this regard to CTZ.

Effect of CX614 on Recombinant AMPA Receptors Expressed in HEK 293 Cells. HEK 293 cells stably expressing recombinant AMPA receptors from rat (Yamada and Turetsky, 1996; Hennegriff et al., 1997) were used to

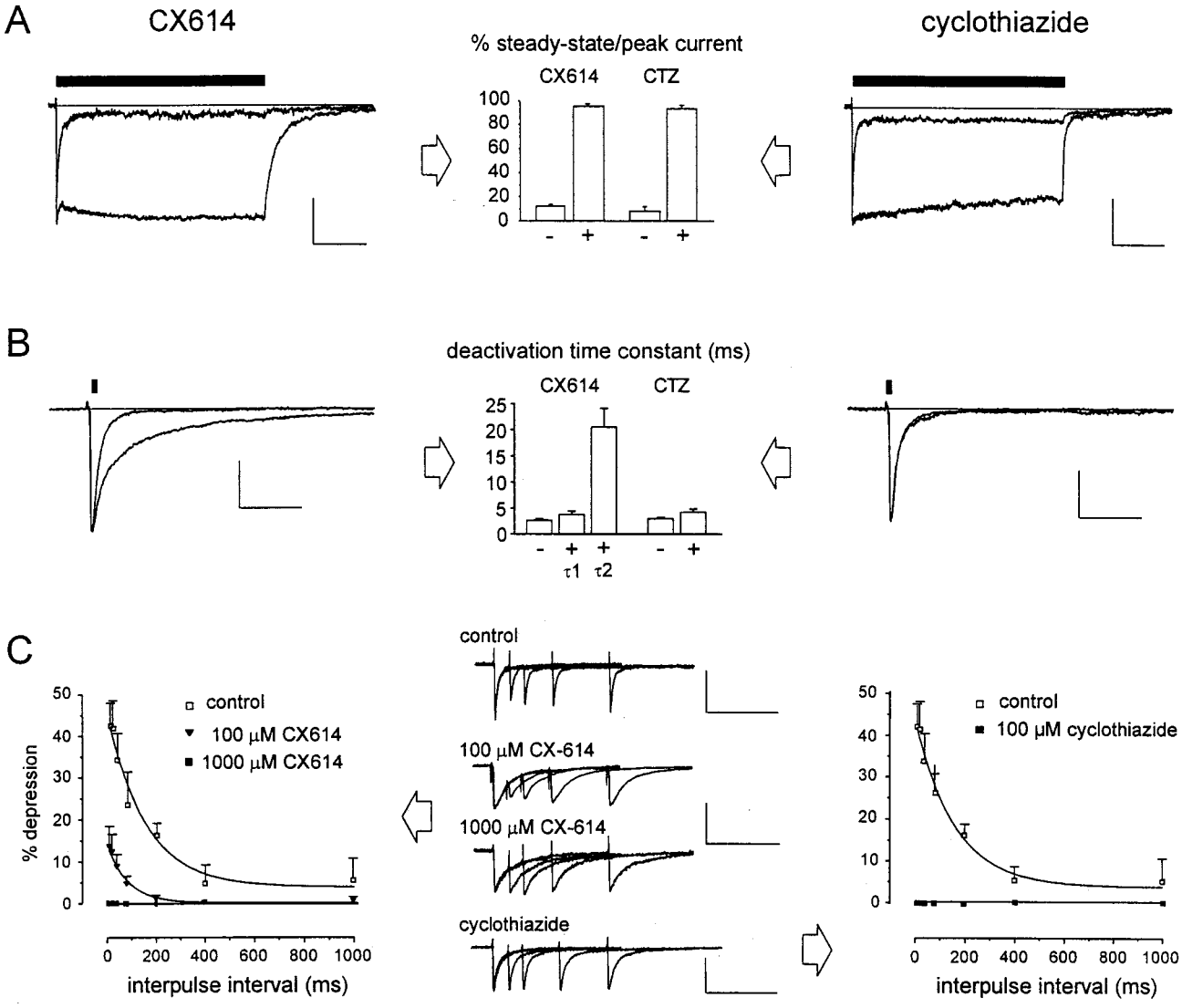

Fig. 5. Effect of CX614 on currents induced by long and short glutamate pulses: comparison with CTZ. Effects of CX614 and CTZ were examined in patches excised from CA1 pyramidal neurons. Patches were equilibrated with the drug before glutamate was applied. A, effects of CX614 and CTZ on inward currents induced by long application of 10 mM glutamate. Traces were taken from a representative experiment in which both drugs were applied at 100 μM to the same patch. Calibration, 50 pA/200 ms for CX614, 40 pA/200 ms for CTZ. The bar graph at the center summarizes the effect of the drugs on the steady-state current as a percentage of the peak current and shows the corresponding control values without drug. Data (mean and S.E.M.) are from 10 (CX614) and 8 patches (CTZ). B, effects on responses induced by 1-ms application of 10 mM glutamate. Traces were taken from the same experiment as in A. Calibration, 50 pA/20 ms. The decay phase was fitted with a single-exponential (control and CTZ) or a two-exponential function (CX614). Summarized data are shown at the center. The deactivation time constant in the absence of drug was 2.6 ± 0.3 ms (n = 8). For CX614, both the fast (τ1) and the slow (τ2) component are shown. The effects of the drugs were compared within the same patches (6 pairs). C, paired-pulse depression. The graphs on the left and right summarize the drug effects on paired-pulse depression. Pairs of 1-ms pulses of glutamate (10 mM) were applied with interpulse intervals between 10 and 1000 ms. The depression in the second response relative to the amplitude of the first response was plotted against the interpulse interval. Each point represents averaged data from five to seven (control), four to five (CX614), and three (CTZ) patches. The data were fitted with a single exponential decay function. Representative experiments with traces for several interpulse intervals superimposed are shown in the middle. Calibration, 50 pA/50 ms for control, 1000 μM CX614 and CTZ; 165 pA/50 ms for 100 μM CX614.

examine whether CX614 possesses a preference for specific subunits or their splice variants "flip" and "flop". Figure 6 shows the effects of CX614 together with those of CTZ on glutamate-induced currents. CX614 increased the peak current in all recombinant receptors tested, the effect in general reaching a maximum at concentrations of 100 to 300 μM; EC_{50} values are listed in Table 1. The potency of CX614 was similar across the three types of flop subunits tested with EC_{50} values ranging from 19 to 37 μM. However, in both flip-flop pairs, the drug showed a modest preference for the flop subunit with values of 37 μM for GluR2 flop and 19 μM for GluR3 flop versus 46 and 71 μM for the respective flip variants. This pattern of preferences, which was reproduced in binding tests, is opposite that of CTZ (Table 1; Hennegriff et al., 1997). Notable differences were also seen in the response kinetics. For CTZ, the apparent affinity correlated with the extent to which desensitization was attenuated in that desensitization was completely blocked in flip subunits, whereas the lower affinity flop subunits exhibited at most a slowing of desensitization. In contrast, CX614 did not completely block the decay of the response of any receptor type and there was no evident relation with the EC_{50} [i.e., the decay of the response at a high CX614 concentration was slower in GluR3 flip (EC_{50} 71 μM) than in GluR1 flop (21 μM) and GluR3 flop (19 μM, not shown) receptors].

Interaction between CX614 and GYKI on AMPA Receptor Currents. If two compounds act through a common site, then the one present in excess relative to its affinity should prevail in a competition situation. To test this, dose-response curves were established for the effect of CX614 on glutamate-induced currents at fixed concentrations of GYKI (Fig. 7). GYKI decreased the peak amplitude of glutamate responses induced by prolonged application by 40% at 10 μM and by 80% at 100 μM (Fig. 7B). Addition of CX614 fully reversed this reduction; i.e., the response amplitudes extrapolated to saturating concentrations of CX614 were comparable with and without GYKI present (Fig. 7, A, bottom, and B). A different result was obtained, however, when the same experiment was carried out with 1-ms applications of glutamate (Fig. 7, E and F). GYKI was effective in reducing the peak glutamate current, as with long glutamate applications, but CX614, even at 2 mM, could not reverse the effects of GYKI to any substantial degree. The discordant results from these two paradigms suggest that there are differences in the drug interactions on the early and later aspects of the responses to prolonged glutamate applications. This was con-

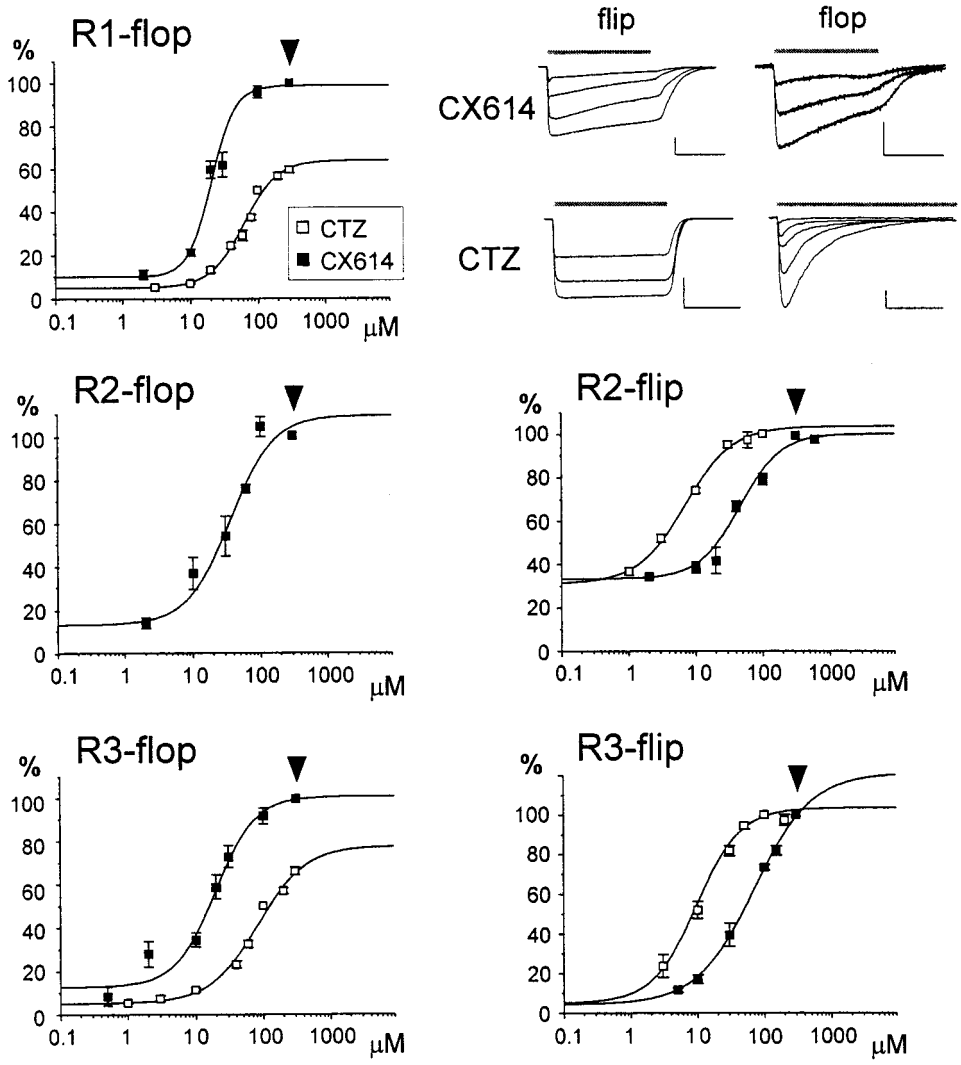

Fig. 6. CX614 dose-response relations for currents through homomeric AMPA receptors. Recombinant, homomeric AMPA receptors were stably expressed in HEK 293 cell lines. Whole-cell currents induced by 10 mM glutamate were measured in the presence of CX614 (filled symbols) or CTZ (open symbols) at the concentrations shown on the *x*-axis. For each cell, peak currents at all drug concentrations tested were normalized to that obtained in the presence of 300 μM CX614, as indicated with arrowheads. EC_{50} values determined by fitting the data points with a logistic equation are summarized in Table 1. The data are averages (mean and S.E.M.) from 4 to 10 (GluR1o), 5 to 19 (GluR2i), 3 to 14 (GluR2o), 3 to 19 (GluR3o), and 5 to 14 (GluR3i) cells. Representative traces for GluR3 flip and GluR1 flop are shown on the upper right. Holding potential, −100 mV. Calibration, 2 nA/500 ms (flip), 200 pA/500 ms (flop).

firmed (Fig. 7C) when responses to long glutamate applications were reanalyzed to determine the amplitude at a fixed latency of 1.2 ms (i.e., at a time when responses in the absence of drug almost reached their peak) (Fig. 7D). GYKI (100 μM) slowed the rise phase significantly (Fig. 7D, open circles) and CX614 was not able to counteract this. The slowing of the rise phase was also observed in the presence of 10 μM GYKI (data not shown). These results thus indicate that the ability of GYKI to block AMPA receptor currents is reduced or eliminated when receptors have bound an ampakine, but that GYKI nevertheless greatly slows the onset of the responses. Evidently, it could not do so unless it was bound to the receptor; thus, one must assume that it binds to a site from which it cannot be displaced by the ampakines.

Interactions of CX614 with GYKI and CTZ: Tests with [^{3}H]Fluorowillardiine Binding. Most AMPA receptor modulators change the binding affinity for agonists such as [^{3}H]AMPA or [^{3}H]fluorowillardiine and the EC_{50} values are generally similar to those in physiological experiments (Hall et al., 1993; Arai et al., 1996b, Kessler et al., 1996). Measuring agonist binding thus provides a method by which to study drug interactions. CX614 by itself produced an increase in the binding of [^{3}H]fluorowillardiine (Fig. 8B) with an EC_{50} value of 88 μM. An increase was also observed for the affinity of glutamate, which was derived from its ability to displace the binding of [^{3}H]CNQX (Fig. 8A). The IC_{50} value for the displacement by glutamate decreased from 44 μM without drug to 12.5 μM at 1 mM CX614; replotting the IC_{50} values against the drug concentrations provides an EC_{50}-value estimate for CX614 of 22 μM. The difference in the EC_{50} from Fig. 8B may be caused by the choice of the agonist (fluorowillardiine versus glutamate) or by the fact that [^{3}H]CNQX displacement preferentially probes the dominant population of low-affinity receptor sites, whereas binding of radiolabeled agonists accords greater weight to the small population of high-affinity sites (Hall et al., 1992).

As in earlier experiments using [^{3}H]AMPA or [^{3}H]CNQX (Kessler et al., 1996), GYKI had a barely detectable influence on [^{3}H]fluorowillardiine binding (+6% at 125 μM). Surprisingly, however, it altered the effect of CX614 in that binding at saturating concentrations of the latter was reduced from 185 to 145% (Fig. 8B). The EC_{50} for CX614 was not significantly altered, which suggests that the interaction between the drugs is not competitive. The same conclusion applies to dose-response curves for GYKI (Fig. 8C), which, regardless of the CX614 concentration, gave EC_{50} values of 15 to 20 μM, a value comparable with that obtained in physiological tests (14 μM; Zorumski et al., 1993).

A more complex pattern of results was obtained for the interaction between CX614 and CTZ (Fig. 9). These tests were carried out with homomeric GluR2 flop receptors because differences in subunit preferences between drugs could mask competition. The incubation temperature was reduced to 0°C because recombinant receptors are unstable at ambient temperature. As in brain membranes, CX614 increased [^{3}H]fluorowillardiine binding, although to a smaller extent. Adding CTZ introduced three types of changes: 1) [^{3}H]fluorowillardiine binding in the absence of CX614 was reduced with an EC_{50} value of 53 μM (see Fig. 9B), which accords with earlier experiments using [^{3}H]AMPA (Hennegriff et al., 1997; Table 1). 2) Although the percentage increase produced by CX614 became larger, the maximum plateau, as determined from the asymptote of the sigmoid curve, declined from >100% to less than 80% (Fig. 9B). 3) The EC_{50} value for CX614 was progressively shifted toward larger values, as is evident from Fig. 9, A and B, inset. Such a shift could occur if two drugs bind to separate sites and allosterically reduce each other's affinity, but then the EC_{50} value for CX614 plotted in the inset should reach a plateau value when CTZ reaches a concentration sufficient to occupy all its sites. Conversely, if the two drugs compete for a single site, then the EC_{50} values for CX614 would be expected to increase linearly with the CTZ concentrations, as indicated by the dashed line. The actual relation shown in the inset suggests a more complex situation, perhaps involving cooperativity among drug sites, but can be reconciled more readily with a competitive interaction. On the other hand, if CX614 and CTZ were competing for a single site, then the plateau of the dose-response curves in Fig. 9A would be expected to remain constant across all CTZ concentrations. The fact that binding at saturating CX614 is depressed by even moderate levels of CTZ suggests that the latter can bind to a site to which CX614 has no access. One likely scenario thus would be that CTZ binds to two distinct types of sites, only one of which is shared with the ampakine. Note that the depression in the asymptote level and the shift in the EC_{50} value were similar for curve fits in which the Hill coefficient was fixed at 1 or allowed to float (Fig. 9B, dotted lines); thus, the conclusions reached above do not seem to depend on particulars of the curve-fitting procedure.

TABLE 1
EC_{50} values for CX614 and CTZ

	CX614	CTZ
	μM	
EPSP		
Amplitude	30	N.D.[a]
Half-width	18	N.D.[a]
Glutamate-induced currents, native receptors from CA1 pyramidal cells[b]		
Peak	132	
Steady-state	43	
% Steady-state/peak	18	10
Glutamate-induced currents, recombinant homomeric receptors		
GluR1o	21	74
GluR2o	37	
GluR2i	46	7
GluR3o	19	78
GluR3i	71	9
[^{3}H]AMPA binding[c]		
GluR1o	20	130
GluR2o	18	49
GluR2i	58	7
GluR3o	40	73
GluR3i	157	16

[a] No EC_{50} values were determined for cyclothiazide because it produced only minor changes in EPSP measures even after prolonged perfusion at 100 μM (Larson et al., 1994; Arai and Lynch, 1998b).
[b] CTZ data for patch currents are from Arai et al. (1996a).
[c] From Hennegriff et al. (1997) for binding data related to GluR1, GluR2, and GluR3 flop. Data for GluR3 flip were determined as in the study of Hennegriff et al. (1997) by measuring the drug effect on the binding of 10 nM [^{3}H]AMPA in the presence of 50 mM potassium thiocyanate at 0°C.

Discussion

The present study introduced an ampakine that is considerably more potent in enhancing synaptic transmission in hippocampal slices than those described in previous reports. Threshold concentration for a detectable increase in the field EPSPs was about 5 μM, and the EC_{50} value was on the order of 20 to 40 μM. These values are about 10 times lower than those reported for CX516 (or BDP-12; Arai et al., 1996b), the ampakine most extensively tested in animals and humans. Increases of 50 to 100% in response amplitude and duration were readily obtained after 20 min of drug infusion, and return to baseline was rapid, as with earlier ampakines. Similar changes in the time course of AMPA receptor-mediated EPSCs were observed in autaptic synaptic responses in primary hippocampal neurons.

A major point of interest is that this action profile is similar for all ampakines, regardless of their potency, yet remarkably distinct from that of CTZ, which, despite its well characterized efficacy in blocking receptor desensitization, produces only modest changes in the wave form of individual field EPSPs. We have reported elsewhere, however, that the effects of these drugs on synaptic responses correlate with their ability to slow deactivation of patch responses to 1-ms glutamate pulses (Arai and Lynch, 1998b). The data shown here confirm this point in that CX614—in keeping with its sizeable effect on synaptic responses—was much more effective than CTZ in slowing deactivation. Taken together, these observations suggest that the decay of fast responses is determined to a much lesser degree by desensitization than by channel closing and transmitter dissociation and that the ampakines more prominently act on the latter. Equilibrium binding studies provided additional evidence in this regard. Receptor affinity was substantially increased by CX614, as would be expected from the idea that the drug decreases deactivation rates by slowing transmitter dissociation or channel closing (Ambros-Ingerson and Lynch, 1993). The *decrease* in binding typically produced by CTZ similarly accords with earlier arguments that the drug shifts AMPA receptor kinetics away from the desensitized state (Hall et al., 1993; Kessler et al., 1996).

EC_{50} values comparable with those in slices and patches were also obtained when CX614 was tested on recombinant,

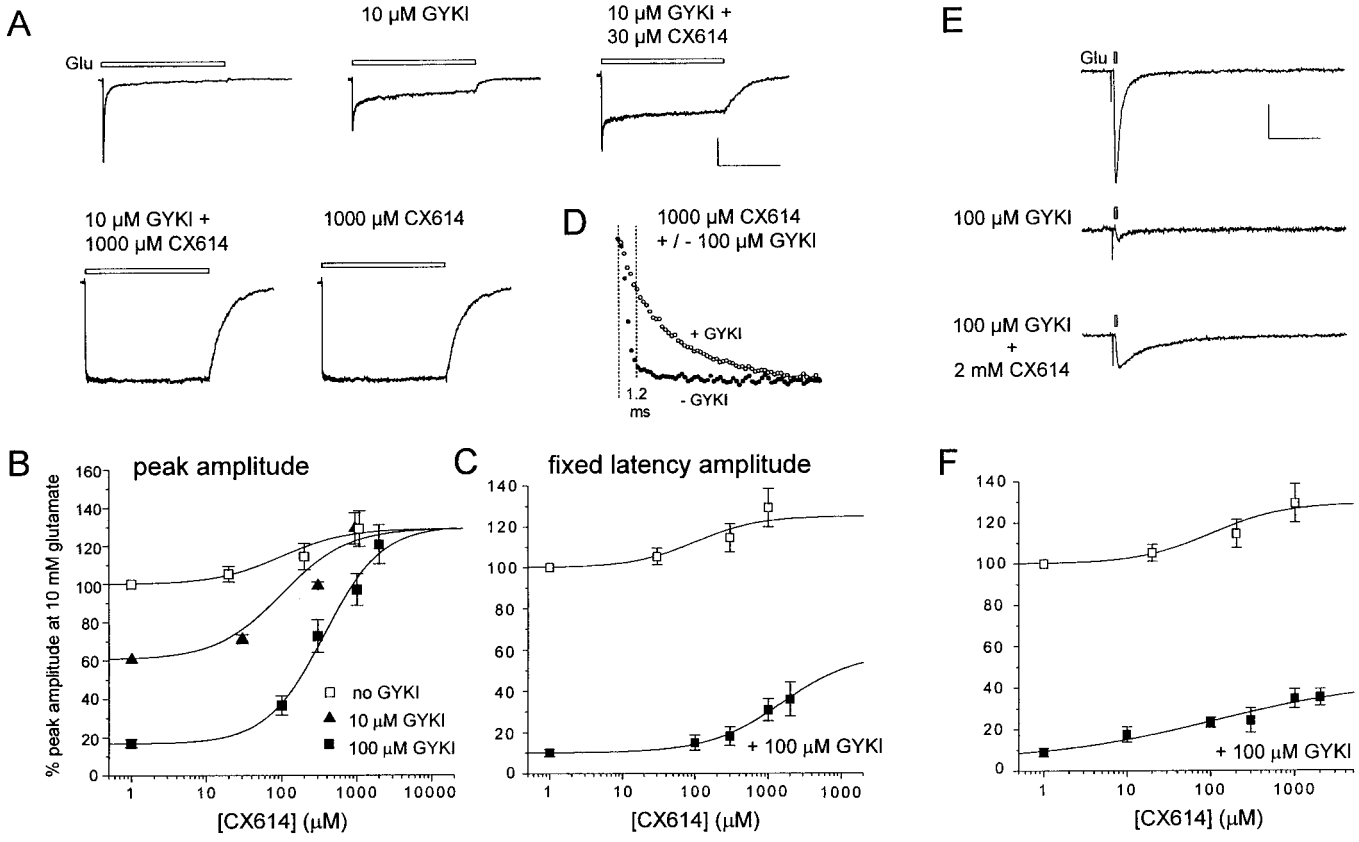

Fig. 7. Interaction between CX614 and GYKI in hippocampal patches. A, interactions in response to a 800-ms application of 10 mM glutamate; representative experiment with traces from a single patch. Responses shown were taken in the absence of drug, in the combined presence of 10 μM GYKI plus 0, 30, or 1000 μM CX614, and in the presence of 1000 μM CX614 without GYKI. Both drugs were always infused for at least 10 s before glutamate application and were present in both background and agonist flow lines. Calibration, 30 pA/400 ms. B, summarized data for the peak amplitude. Patches were equilibrated with fixed concentrations of GYKI (0, 10, and 100 μM) and various concentrations of CX614. The peak amplitude was plotted against the concentration of CX614. Each point represents the mean and S.E.M. of three to six (CX614 alone), four to six (10 μM GYKI + CX614), and five to eight patches (100 μM GYKI + CX614). C, summarized data for the amplitude measured at a fixed latency of 1.2 ms after response onset. Data are from the same recordings as in B (0 and 100 μM GYKI); amplitudes were plotted against the concentration of CX614. D, effects of GYKI (100 μM) on the rising phase of glutamate-mediated responses in the presence of 1000 μM CX614. The vertical lines indicate the time of response onset (t = 0) and the 1.2-ms time point at which the amplitudes were measured that are plotted in C. E, interactions in responses to 1-ms pulses of 10 mM glutamate. Representative traces from a single patch, recorded in the absence of drug and in the presence of 100 μM GYKI without and with 2 mM CX614. Calibration, 50 pA/20 ms. F, summarized data for experiments as shown in E. Peak amplitudes in the presence of 0 or 100 μM GYKI were plotted against the concentration of CX614. Each point represents data (mean and S.E.M.) from three to eight patches.

homomeric AMPA receptors. The ampakine did not have strong subunit preferences but seemed to prefer the flop over the corresponding flip variants. This pattern, which was seen in both physiological and binding tests, is similar to that reported for aniracetam (Tsuzuki et al., 1992; Partin et al., 1996) and distinct from that of CTZ, which, as in previous studies (Partin et al., 1994), had a 5- to 10-fold higher affinity for flip isoforms. Also, CTZ exhibited an obvious relationship

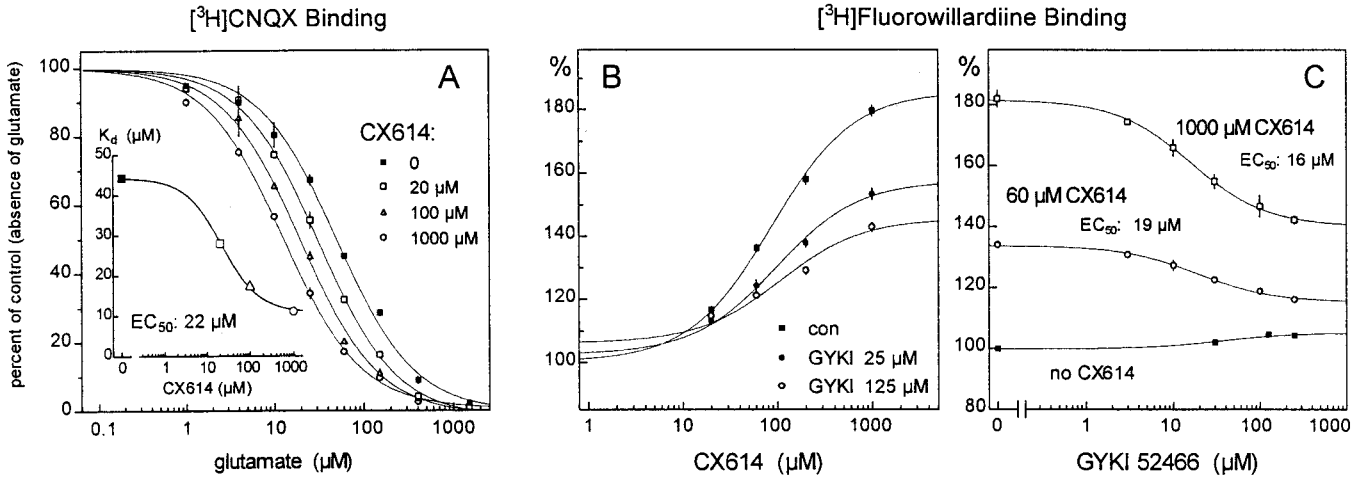

Fig. 8. Effect of CX614 on agonist binding to AMPA receptors; lack of competition with GYKI. A, displacement of [^{3}H]CNQX binding by glutamate in the absence of CX614 and in the presence of 20, 100, and 1000 μM CX614. Binding was measured at 25°C with a centrifugation assay. Rat brain membranes were incubated for 60 min at 25°C with 40 nM [^{3}H]CNQX (without thiocyanate) and the glutamate concentrations indicated on the x-axis. CX614 was added from 100-fold concentrated solutions in DMSO; control cells received 1% DMSO. Data points and error bars represent means and S.E.M. from two to four experiments at each drug concentration. Sigmoidal dose response curves (logistic equation) with $n_H = 1$ were fitted to the data points using nonlinear regression; the curves were forced through 100 and 0% because the corresponding measurements (at 0 and 5 mM glutamate) were made with twice the number of replicates that were used for the other points. The single-site fits could not accommodate the small but evident deviations at low glutamate concentrations because of the presence of high-affinity sites (Hall et al., 1992); the fits are therefore representative mainly of the prevalent low affinity population of receptors. The IC_{50} values for glutamate obtained from these curves were converted to K_d values by multiplying the IC_{50} with a Cheng-Prusoff factor of 0.89 (K_D for [^{3}H]CNQX under assay condition ~300 nM; Kessler et al., 1996) and replotted in the inset graph against the CX614 concentration. The EC_{50} value for CX614 determined from this concentration-effect curve is 22 μM. B, effect of CX614 on [^{3}H]fluorowillardiine binding (20 nM; no thiocyanate); concentration-effect curves in the presence of 0, 25, and 125 μM GYKI. Assays and data analysis procedures were as described above. GYKI was added from 200-fold concentrated solutions in DMSO; all incubations were matched in DMSO. EC_{50} values for CX614 at 0, 25, and 125 μM GYKI were 88, 109, and 98 μM, respectively; additional determinations at 250 μM GYKI were not detectably different from those at 125 μM (not shown). C, concentration-effect curves for GYKI in the presence of 0, 60, and 1000 μM CX614. No EC_{50} value was determined for the case without CX614, but a previous determination using [^{3}H]CNQX as radioligand provided an EC_{50} of 16 μM (Kessler et al., 1996).

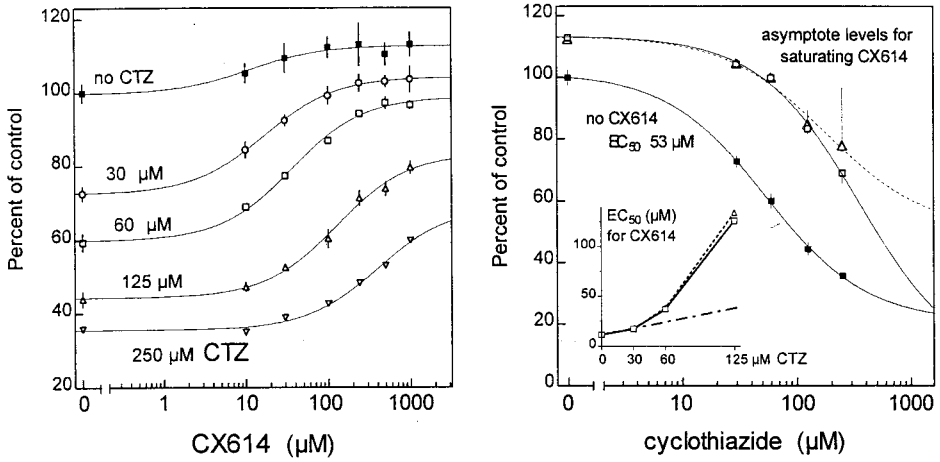

Fig. 9. [^{3}H]Fluorowillardiine binding to homomeric GluR2-flop receptors: interaction between CX614 and CTZ. Left, concentration-effect curves for CX614 were measured in the presence of 0, 30, 60, 125, and 250 μM CTZ, using a filtration assay. Membranes from HEK 293 cells expressing GluR2-flop receptors were incubated for 60 min at 0°C with 10 nM [^{3}H]fluorowillardiine and the indicated drug concentrations (final DMSO concentration in all samples, 0.9%). Data are averages (mean and S.D.) from two to six determinations, normalized within each experiment to the average binding value obtained in the absence of any drug. Curves were fitted to the data points using logistic equations with $n_H = 1$. Almost identical curves were obtained when Hill coefficients were allowed to float. Right, lower and upper asymptotes of the sigmoidal curves on the left are replotted against the CTZ concentration. Plotting of binding values at 0 μM CX614 provides an EC_{50} estimate for CTZ of 53 μM (filled symbols). The upper asymptotes, which represent binding at saturating concentrations of CX614, are shown with open symbols; data from fits with an $n_H = 1$ are indicated by squares; those in which the Hill coefficient was allowed to float are indicated by triangles. EC_{50} values for the later cases are between 180 to 350 μM. Inset, the EC_{50} values of the curves fitted on the left were plotted against the concentration of CTZ. Data from fits with an $n_H = 1$ are shown with squares, those with variable Hill coefficient with triangles. EC_{50} values at 250 μM CTZ (400–700 μM) were omitted. The dashed line indicates the relation expected for a simple competitive type interaction in which CX614 has an EC_{50} value of 11.3 μM and CTZ has an EC_{50} value of 53 μM.

between affinity and efficacy in blocking desensitization, but no such relation was seen with CX614. This may indicate again that the ampakine influenced aspects of receptor kinetics other than or in addition to desensitization.

The competition experiments led to the conclusion that the modulators examined in this study most probably act through three distinct sites (Fig. 10). Both binding and physiological tests clearly indicated lack of competition between CX614 and GYKI, despite partial structural homology. In the patch experiments, CX614 could not reverse the inhibition by GYKI of brief glutamate responses. With long glutamate applications, CX614 seemed to reverse the inhibitory effects of GYKI, but closer inspection of the traces showed that the currents measured at high GYKI + CX614 concentrations had a greatly increased rise time, which evidently means that both drugs were acting simultaneously on the same receptor. A delay of the peak onset by GYKI has been shown in other studies with heteromeric recombinant receptors (Johansen et al., 1995) and native AMPA receptors (Rammes et al., 1996). A plausible explanation would be that GYKI slows either channel opening or the association of glutamate with the receptor, as suggested in the latter report. Because changes in the association rate should greatly alter binding affinity (Ambros-Ingerson and Lynch, 1993) and GYKI, when given alone, did not change binding, a slowing of channel opening seems to be the most likely effect of the drug.

The interaction between CX614 and GYKI thus evidently occurs at the level of receptor kinetics by acting on a shared set of kinetic parameters. A plausible scenario would be that CX614 prominently slows channel closing and that this can effectively counteract the slowing of channel opening caused by GYKI, provided that glutamate is present long enough that a steady state can be reached. It would also provide a satisfactory interpretation for the binding results of Fig. 8. Calculations using an equation for the binding K_D in a five-state receptor model (Ambros-Ingerson and Lynch, 1993) readily show that a slowing of channel closing by CX614 would significantly increase binding affinity, whereas a slowing of channel opening by GYKI would have negligible effects on agonist binding but would effectively oppose any affinity increase caused by a drug that slows channel closing. That GYKI did not reverse the binding changes caused by CTZ (Kessler et al., 1996) again would agree with the notion that CTZ acts more prominently on desensitization kinetics rather than on aspects of channel gating.

Interactions between CX614 and CTZ were of more complex nature, suggesting in the end that they act through two separate sites, one of which would bind both drugs, the other one being rather selective for CTZ. Because we employed homomeric receptors in which all subunits are identical, the two sites cannot be homologous loci on different subunits. It cannot be ruled out, of course, that other forms of receptor heterogeneity exist, perhaps caused by different degrees of post-translational modification (such as phosphorylation). No such influences on drug affinity have been described, however, nor would they be likely to explain the data of Fig. 9A unless binding of CX614 would be controlled in an all-or-none fashion. Thus, the presence of two distinct sites for up-modulatory drugs on each subunit remains the most probable explanation. The suggested interactions of CX614, CTZ, and GYKI with the receptor and the hypothesized kinetic targets are summarized in Fig. 10. CX614 is proposed to have a mixed influence on desensitization and channel gating kinetics, reflecting its efficacy in completely blocking desensitization and its ability to prolong response deactivation more than 8-fold. Cyclothiazide may share some of these effects; however, by virtue of its binding to a second site more closely linked to desensitization, it may act more prominently on the latter aspect of receptor kinetics.

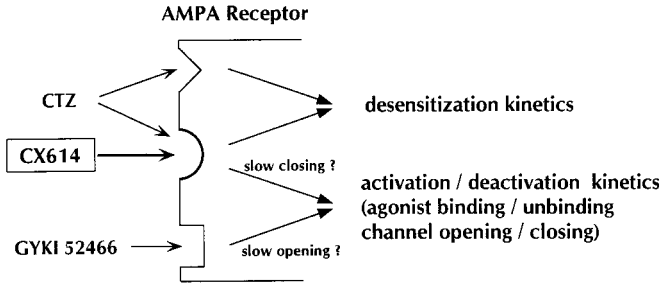

Fig. 10. Summary of proposed drug-receptor interactions

In conclusion, changes in ampakine design to confer structural rigidity increased potency by at least an order of magnitude without markedly changing the modulatory actions found in earlier drugs. Like BDP (Arai et al., 1994) and BDP-20 (Arai et al., 1996a), CX614 increased the amplitude and especially the duration of field EPSPs, slowed receptor deactivation, and increased the affinity of AMPA receptors for agonists. Thus, the contrast between the effects of ampakines and those of CTZ can no longer be ascribed to any gradient in potency and points instead to a different action on receptor kinetics. Drug interaction experiments suggested that AMPA receptors may indeed have at least two sites for up-modulators, in addition to a site for down-modulators. With further improvements in the potency of the modulatory compounds, we hope that it will be possible to study the identity of these sites using radiolabeled variants of the drugs.

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