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Negative Allosteric Modulation of Wild-Type and Mutant AMPA Receptors by GYKI 53655

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SUMMARY

Benzothiadiazides such as cyclothiazide potentiate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor responses, whereas 2,3-benzodiazepines such as 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine (GYKI 53655) act as noncompetitive antagonists; both drugs act through allosteric modulation. Controversy exists as to whether cyclothiazide and GYKI 53655 act at a common site. Recent mutational analysis has led to the identification of a serine residue in flip splice variants that is critical for directing the interaction of cyclothiazide with AMPA receptors. We tested whether the mutation of this residue to glutamine, which abolishes potentiation by cyclothiazide, can in addition block antagonism by 2,3-benzodiazepines, as would be predicted for action at a common site. We found that the S to Q mutation does not alter antagonism by 2,3-benzodiazepines, suggesting that the molecular determinants directing the interaction between GYKI 53655 and AMPA receptors are not identical to those controlling sensitivity to cyclothiazide. Additional support for this was obtained from analysis of the responses of AMPA receptor flip/flop splice variants, which, despite differences in equilibrium desensitization and sensitivity to cyclothiazide, show only small differences in sensitivity to 2,3-benzodiazepines. Furthermore, introduction of the flip exon from GluRA into GluR6, conferred sensitivity to cyclothiazide but did not increase sensitivity to 2,3-benzodiazepines. Of interest, experiments with native AMPA receptors generated from hippocampal and forebrain poly(A)$^+$ mRNA revealed greater sensitivity to 2,3-benzodiazepines than receptors generated by expression of recombinant AMPA receptors, possibly indicating the existence of an unidentified accessory protein or novel receptor subunit.

Neurons and glia express multiple subtypes of non-NMDA glutamate receptor ion channels that are formed by subunits from the AMPA (GluRA-D or GluR1–4), kainate (GluR5–7), and high affinity kainate binding protein (KA-1 and KA-2) gene families (1, 2). Although there is evidence that AMPA and kainate receptor subunits are coexpressed within single cells, electrophysiological and immunological data suggest that there is unlikely to be functional coassembly of AMPA and kainate receptor subunits (3–7). AMPA and kainate receptors show both positive and negative allosteric regulation. Cyclothiazide potentiates responses at AMPA receptors, at least in part by blocking the onset of desensitization, a conformational transition to a closed state in the continued presence of agonist (8, 9). Cyclothiazide exhibits a very high degree of selectivity, acting exclusively on AMPA but not kainate receptors (10, 11), and more potently modulates flip versus flop splice isoforms (12).

A group of structurally related 2,3-benzodiazepines act as reversible noncompetitive antagonists at native AMPA receptors (13, 14), with a similar but much less potent action at native kainate receptors (15, 16). The molecular mechanism(s) underlying AMPA receptor antagonism by 2,3-benzodiazepines such as GYKI 52466 and GYKI 53655 have not been determined, although it has been shown that GYKI 52466 does not alter the extent or kinetics of onset of desensitization, nor does it act as an open channel blocker, and therefore must be acting through negative allosteric modulation of the receptor (13). 2,3-benzodiazepines have anticonvulsant activity in seizure paradigms, presumably through attenuation of AMPA receptor-mediated excitatory synaptic transmission, which makes these compounds potentially useful as therapeutic anticonvulsants (17). To address the mechanism of action of 2,3-benzodiazepines, several laboratories have posed the question of whether GYKI 52466 and cyclothiazide act via common mechanisms (14, 18–20). The interpretation of these experiments is controversial in that different groups have found that there is no interaction between these drugs (18, 19) or that cyclothiazide and 2,3-benzodiazepines reverse the action of each other (14, 20). One confounding parameter of these experiments is that they

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DMSO, dimethylsulfoxide; GluR, glutamate receptor subunit; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; GYKI 53655, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansesulfonic acid; PCR, polymerase chain reaction.

were performed in a variety of preparations, with possibly different expression of AMPA receptor subtypes; in addition, in some experiments only indirect assays of AMPA receptor activity were performed.

We recently described a mutant of an AMPA receptor flip splice variant, GluRA$_i$[S$_{750}$Q], that completely abolishes modulation by cyclothiazide but has no effect on activation or desensitization of control responses to glutamate, indicating that in all respects other than modulation by cyclothiazide the mutation is silent (21). If this mutation blocks antagonism by 2,3-benzodiazepines, this would support the proposal that the molecular determinants of benzodiazepines and cyclothiazide are identical (14). A second approach used to address this issue, based on previous experiments in which we described a point mutation which conferred sensitivity to cyclothiazide in kainate receptors (21), was to generate a kainate/AMPA receptor chimera by introducing the flip exon from GluRA into GluR6. The results of these experiments do not support the hypothesis that cyclothiazide and 2,3-benzodiazepines produce allosteric regulation via identical molecular determinants.

Materials and Methods

Plasmids and mutants. Plasmids encoding cDNAs for the flip (i) and flop (o) variants of wild-type GluRA$_i$, GluRA$_o$, GluRB$_i$, GluRB$_o$, GluRD$_o$, and GluR6 were gifts of Dr. Peter Seeburg (University of Heidelberg, Germany). Glutamate receptor point mutants were made as described previously (21). The GluR6/1 chimera was made by first introducing into GluR6 unique restriction sites that flanked the region of interest (*Stu*I and *Nhe*I, Fig. 1A) using *dut-ung-* missense mutagenesis. PCR mutagenesis with primers generating compatible ends was then used to amplify the GluRA$_i$ sequence around the flip exon; the PCR product was then subcloned into the *Stu*I and *Nhe*I sites in GluR6. DNA sequencing was performed to confirm the sequence of the region that was manipulated. The wild-type and mutant glutamate receptor cDNAs were subcloned into a high expression *Xenopus* oocyte vector, pGEMHE, a gift from Dr. Emily Liman (Massachusetts General Hospital, Boston, MA). All DNA preparations were made by alkaline lysis followed by cesium chloride gradient purification.

Oocyte expression. Capped mRNA was synthesized *in vitro* from AMPA receptor cDNAs using T7 polymerase (mMessage Machine, Ambion). Poly(A)$^+$ mRNA was purified from total RNA that had been isolated from adult rat hippocampus or P10 forebrain using guanidinium isothiocyanate (22) and purified using PolyAQuik columns (Stratagene). The final concentration of RNA preparations was quantified by spectroscopy. Oocytes were surgically obtained from anesthetized adult *Xenopus laevis* (Nasco) and prepared as previously described (10). Then, 27.5 or 50 nl of RNA at 0.5–1.0 μg/μl was injected into the oocyte with the use of a Drummond positive displacement injector. For heteromeric combinations, a 1:5 ratio of GluRA/GluRB was used to force hetero-oligomerization. Within every experiment, some oocytes were tested to be certain that linear current-voltage curves were obtained. Oocytes were incubated at 18° in Barth's solution containing 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, for 2–6 days before recording.

Oocyte electrophysiology. Two-electrode voltage-clamp experiments were performed as previously described (12, 21). Large (1–10 μA) currents required the use of a virtual ground headstage to clamp the bath potential at ground, as well as low-resistance agarose-cushion recording electrodes (23). The oocyte was continuously perfused with a modified Barth's solution that replaced calcium with barium; kainate (Sigma), cyclothiazide (a gift of Eli Lily made up as a 20 mM stock in DMSO), GYKI 52466 or GYKI 53655 (gifts of Eli Lilly made up as 5 mM stocks in DMSO) were added as required. DMSO was added to solutions lacking cyclothiazide and/or 2,3-benzodiazepines so that all solutions contained equivalent amounts of vehicle. Electrophysiological responses were acquired by an ITC-16 interface (Instrutech Corp.) under the control of a data acquisition and analysis program (Synapse, Synergistic Research Systems)[1] run on a MacIntosh IIfx. Dose-response analysis for inhibition by 2,3-benzodiazepines was performed using 300 μM kainate with or without 100 μM cyclothiazide. Responses were fit with a logistic equation:

$$I = I_{max} \times (1/[1 + (EC_{50}/[ligand])^n)]$$

where I_{max} is the response at a saturating concentration of ligand, EC_{50} the concentration of ligand producing a half-maximal response, and n is the Hill coefficient. To allow for correction of rundown, bracketing responses to a given concentration of kainate were recorded at the beginning and end of the dose-response run, and then the dose-response curve for a 2,3-benzodiazepine plus kainate was collected, also with bracketing responses. A linear function describing the time course of rundown was fit to the bracketing responses for each oocyte, and then the experimental responses were normalized to the interpolated control value. All values are presented as the mean ± standard error. Tests for statistical significance were performed using unpaired t tests or analysis of variance.

Results

Inhibition by GYKI 53655 of a cyclothiazide-insensitive AMPA receptor. Previous experiments have demonstrated that for homomeric AMPA receptors, the mutation GluRA$_i$[S$_{750}$Q] is sufficient to abolish potentiation by cyclothiazide of responses to both kainate and glutamate (21). The GluRA$_i$[S$_{750}$Q] mutant also fails to show block of desensitization by cyclothiazide or slowing of the time constant of onset of desensitization by cyclothiazide. The GluRA$_i$[S$_{750}$Q] mutation thus is ideally suited to determine whether cyclothiazide acts at the same site as the 2,3-benzodiazepines GYKI 52466 and GYKI 53655, two structurally related antagonists with different potencies at native AMPA receptors (15, 17). To test this, we expressed in *Xenopus* oocytes hetero-oligomers generated from GluRA$_i$[S$_{750}$Q] and GluRB$_i$[S$_{754}$Q], mutant AMPA receptor subunits that generate a receptor that similar to homomeric GluRA$_i$[S$_{750}$Q], does not show modulation by cyclothiazide (Fig. 1). Control responses evoked by 0.3 mM kainate were decreased by 27.6 ± 1.3% with 30 μM GYKI 52466 and by 77.1 ± 1.0% with 30 μM GYKI 53655 (six experiments). At these concentrations, GYKI 52466 and GYKI 53365 produce ~70% and ~95% inhibition in hippocampal and cortical neurons (13–15, 17). Similar to the behavior of homomeric GluRA$_i$[S$_{750}$Q], responses evoked by kainate for GluRA$_i$[S$_{750}$Q] GluRB$_i$[S$_{754}$Q] did not show any potentiation by cyclothiazide (kainate + cyclothiazide/kainate = 0.92 ± 0.01); in the presence of cyclothiazide, kainate-evoked currents were decreased by 31.7 ± 1.1% with 30 μM GYKI 52466 and by 78.0 ± 0.9% with 30 μM GYKI 53655, comparable to the levels of antagonism recorded in control solution. Dose-response analysis for inhibition by 2,3-benzodiazepines revealed an IC_{50} of 10.4 ± 0.7 μM for inhibition by GYKI 53655; in the presence of 100 μM cyclothiazide, the IC_{50} of 10.0 ± 0.3 μM showed no significant difference from control. Our conclusion from these experiments is that abolishing AMPA receptor modulation by cy-

[1] Available by FTP from zippy.nimh.nih.gov in the directory/pub/synapse.

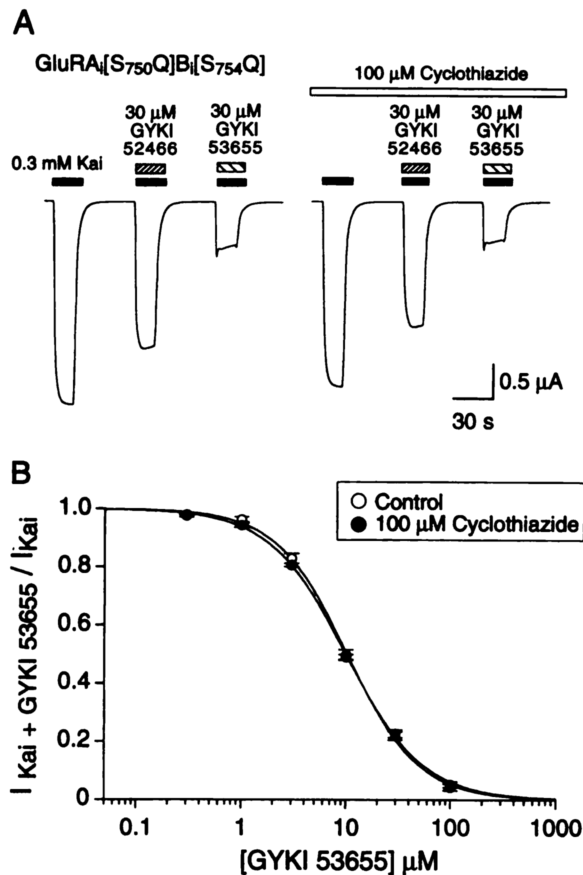

Fig. 1. AMPA receptor mutants insensitive to cyclothiazide are antagonized by GYKI 52466 and 53655. A, Inward currents evoked by 300 μM kainate from a *Xenopus* oocyte expressing GluRA$_i$[S$_{750}$Q] and GluRB$_i$[S$_{754}$Q] hetero-oligomers. Due to the S to Q point mutations, responses do not show potentiation by 100 μM cyclothiazide. However, both 30 μM GYKI 52466 and 30 μM GYKI 53655 antagonize kainate responses to a similar extent in either control solution or with 100 μM cyclothiazide. Transients visible during responses to GYKI 53655 reflect simultaneous application of agonist and antagonist at the beginning of the response to kainate and relief of block before decay of the response to agonist after switching into control solution. B, Mean responses to 300 μM kainate in the presence of increasing concentrations of GYKI 53655 normalized to maximum responses (data points show mean ± standard error). IC$_{50}$ values estimated from a logistic equation fit to the mean responses were 10.0 μM in control and 10.3 μM in 100 μM cyclothiazide (four experiments).

clothiazide did not prevent antagonism by 2,3-benzodiazepines, and therefore these two classes of compounds direct allosteric regulation via different molecular determinants.

2,3-Benzodiazepine inhibition of recombinant AMPA receptor splice variants. Although the above experiment clearly demonstrates that an amino acid that critically directs AMPA receptor modulation by cyclothiazide is not necessary for directing antagonism by 2,3-benzodiazepines, the potency of block by GYKI 53655, IC$_{50}$ 10.4μM, was substantially less than that reported in studies using native AMPA receptors expressed in hippocampal or cortical neurons, IC$_{50}$ of 1.1 and 0.8μM, respectively (15, 17). One interpretation of the reduced potency of 2,3-benzodiazepines for heteromers generated from GluRA$_i$[S$_{750}$Q] and GluRB$_i$[S$_{754}$Q] could be that the position 750 point mutations in GluRA and GluRB had a deleterious effect on 2,3-benzodiazepine antagonism, independent of the presence or absence of cyclothiazide. To test this, we expressed hetero-oligomeric combinations of the flip splice variant of wild-type AMPA receptors generated by assembly of GluRA$_i$ with GluRB$_i$ (Fig. 2). In the absence of cyclothiazide, kainate responses for GluRA$_i$-GluRB$_i$ were decreased 22.0 ± 2.2% with 30 μM GYKI 52466 and 73.4 ± 1.7% by GYKI 53655 (10 experiments), similar to results obtained for the mutant GluRA$_i$[S$_{750}$Q] GluRB$_i$[S$_{754}$Q], indicating that the S to Q mutation does not underlie the lower potency of 2,3-benzodiazepines compared with results obtained with native AMPA receptors (15, 17).

Another potential mechanism that might account for the higher potency of inhibition by 2,3-benzodiazepines in experiments on cortical and hippocampal neurons would be an effect of alternative splicing on AMPA receptor sensitivity to noncompetitive antagonists. Because GluRA$_i$B$_i$ and GluRA$_o$B$_o$ show clear differences in extent of equilibrium desensitization (21, 24, 25), one might have predicted that flip and flop splice variants would be differentially modulated by 2,3-benzodiazepines if these drugs bind with higher affinity to the desensitized state of glutamate receptors. Although the extent of inhibition by 30 μM GYKI 52466 and GYKI 53655 for kainate responses recorded from GluRA$_o$B$_o$ (33.6 ± 1.8% and 78.7 ± 0.8%, five experiments) was slightly greater than that for GluRA$_i$B$_i$ (Fig. 2, A and B), the inhibitory effect on flop splice variants was still weaker than that observed for native AMPA receptors.

One striking difference in the behavior of wild-type AMPA receptors generated from GluRA and GluRB compared with results obtained for GluRA$_i$[S$_{750}$Q] GluRB$_i$[S$_{754}$Q] was a reduction in potency of inhibition by 2,3-benzodiazepines in the presence of cyclothiazide. In the presence of 100 μM cyclothiazide, inhibition by GYKI 53655 was 61.8 ± 3.3% for GluRA$_i$B$_i$ and 73.2 ± 2.5% for GluRA$_o$B$_o$; thus, the ratio (cyclothiazide + GYKI 53655)/cyclothiazide for kainate-evoked currents was 0.84 ± 0.03 for GluRA$_i$B$_i$ and 0.93 ± 0.02 for GluRA$_o$B$_o$. To more fully characterize the action of GYKI 53655 on wild-type recombinant AMPA receptors, we performed dose-inhibition analysis for GluRA$_i$B$_i$ and GluRA$_o$B$_o$ (Fig. 1, C and D). In control solution, GYKI 53655 had an IC$_{50}$ of 11.3 ± 0.7 μM for GluRA$_i$B$_i$ and an IC$_{50}$ of 9.7 ± 0.1 μM for GluRA$_o$B$_o$. These values are essentially identical to those reported for GluRA$_i$[S$_{750}$Q] GluRB$_i$[S$_{754}$Q] and are ~10-fold lower than those reported for native AMPA receptors (15, 17). In the presence of 100 μM cyclothiazide, the IC$_{50}$ values for wild-type GluRA$_i$B$_i$ were 22.3 ± 3.0 μM and 17.8 ± 1.9 μM, respectively; the 2-fold shift is also less than that observed in experiments on native receptors (14).

For the experiments described above, we used hetero-oligomeric combinations of GluRA and GluRB because it is thought that many native neuronal populations are composed of hetero-oligomeric complexes that include these subunits (6, 26, 27). To test whether an interaction between the GluRA and GluRB subunits might affect allosteric modulation by 2,3-benzodiazepines, we performed experiments with homomeric GluRA. In control solution, 30 μM GYKI 53655 inhibited responses to 300 μM kainate by 79.9 ± 2.7%; in 100 μM cyclothiazide, 30 μM GYKI 53655 inhibited responses by 70.8 ± 2.9% (six experiments). The similarity between the potency of GYKI 53655 on homomeric versus hetero-oligomeric recombinant receptors suggests that antagonism by

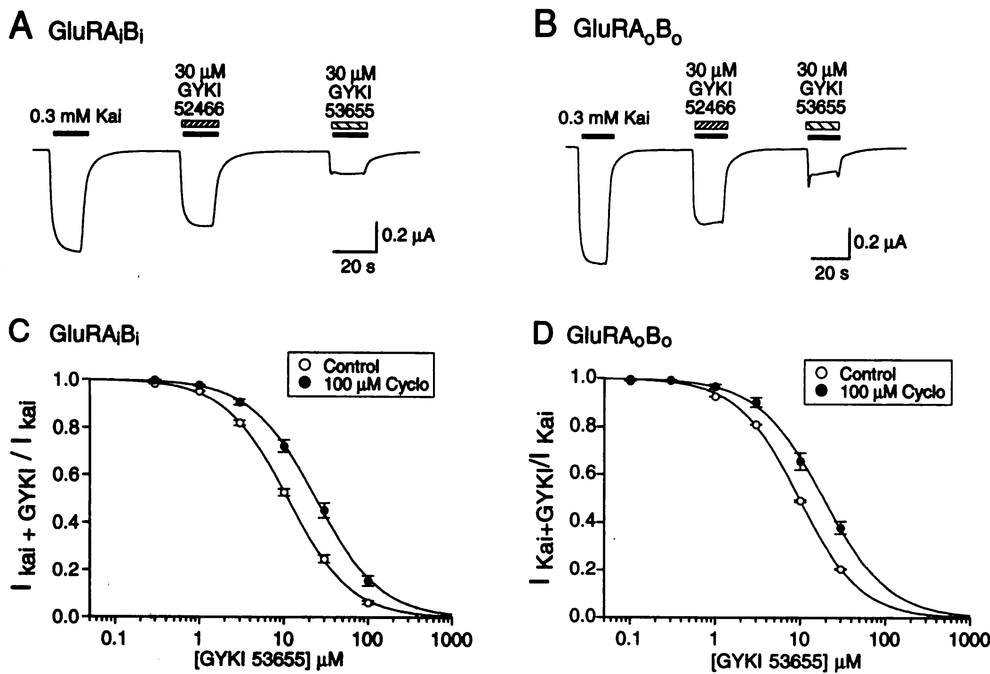

Fig. 2. Flip/flop splice variants do not differ appreciably in sensitivity to antagonism by GYKI 52466 or 53655. Inward current responses to 300 μM kainate for oocytes expressing GluR-A_iB_i (A) or $GluRA_oB_o$ (B) hetero-oligomers, either in control solution or with 30 μM GYKI 52466 or GYKI 53655. These 2,3-benzodiazepines antagonize kainate responses to a similar extent, regardless of the expression of either the flip or flop exon. Dose-inhibition analysis performed as in Fig. 2 reveals for Glu-RA_iB_i (C) a mean IC_{50} of 11.1 μM in control solution (○, five experiments) and 24.0 μM in 100 μM cyclothiazide (●, three experiments); for $GluRA_oB_o$ (D), these values are 9.7 μM and 18.8 μM, respectively.

2,3-benzodiazepines appears not to be sensitive to the hetero-oligomeric state of the receptor complex.

A GluR6/GluR1 chimera sensitive to cyclothiazide but insensitive to 2,3-benzodiazepines. The above experiments demonstrate that block of potentiation by cyclothiazide in the point mutant $GluRA_i[S_{750}Q]$ does not eliminate antagonism by 2,3-benzodiazepines. Although the flip and flop splice variants of AMPA receptor hetero-oligomers generated from GluRA and GluRB do not differ in sensitivity to 2,3-benzodiazepines, it is possible that lack of an effect of the point mutant $GluRA_i[S_{750}Q]$ on sensitivity to 2,3-benzodiazepines could occur because other amino acids within the flip/flop region contribute to binding of these antagonists. To test this, we took advantage of the selectivity of 2,3-benzodiazepines for AMPA versus kainate receptors (15, 16) and constructed a GluR6/GluRA chimera (GluR6/1) that introduced the entire flip exon of GluRA into GluR6 (Fig. 3A). Fig. 3B shows that similar to native kainate receptors in DRG neurons, responses for wild-type GluR6 are not strongly antagonized by 30 μM GYKI 52466 or GYKI 53655 ($I_{GYKI}/I_{Control}$, 1.01 ± 0.02, six experiments) either in control solution or with 100 μM cyclothiazide. Fig. 3C shows that the GluR6/1 chimera is sensitive to modulation by cyclothiazide. On average, there was 26-fold potentiation of peak responses to kainate, presumably due to slowing of the rate of onset of desensitization, as described previously for 293 cells transfected with the point mutant $GluR6[Q_{755}S]$ (21); slow solution exchange in oocyte experiments prevented accurate measurement of the effects of cyclothiazide on the kinetics of desensitization for the GluR6/1 chimera. Similar to wild-type GluR6, 2,3-benzodiazepines fail to antagonize either control responses or cyclothiazide-potentiated responses of the GluR6/1 chimera ($I_{GYKI}/I_{Control}$, 0.95 ± 0.02, five experiments). We therefore conclude that the flip/flop domain does not encode amino acids that are sufficient to direct the selective action of 2,3-benzodiazepines on AMPA receptors. The point mutant, $GluR6[Q_{755}S]$, similarly did not permit antagonism by 2,3-benzodiazepines (data not shown).

Potent antagonism by 2,3-benzodiazepines of AMPA receptors encoded by rat forebrain mRNA. Because we observed only a weak effect of sequence heterogeneity in the flip/flop region on sensitivity to antagonism of recombinant AMPA receptors by 2,3-benzodiazepines, it appeared improbable that the greater sensitivity observed in experiments on native glutamate receptors reflected differences in alternative splicing of the flip/flop domain. Other possibilities we considered were (i) failure to supply a subunit other than GluRA or GluRB but necessary for potent antagonism by 2,3-benzodiazepines, (ii) failure of oocytes to assemble a receptor with a subunit stoichiometry necessary for high sensitivity to 2,3-benzodiazepines, and (iii) failure of oocytes to correctly glycosylate or otherwise post-translationally modify AMPA receptors in a manner that is required for full antagonism. In an attempt to test these issues, we coinjected a combination of $GluRA_i/A_o/B_i/B_o/D_o$ into oocytes at a ratio of 1:1:5:5:1 based on data from reverse PCR of cultured hippocampal neuron total RNA (using primers specific for each AMPA receptor subunit; data not shown) that suggested these to be predominant AMPA receptor mRNA species. Again, the extent of antagonism by 2,3-benzodiazepines was similar to that for hetero-oligomers generated from GluRA and GluRB, indicating that additional subunits failed to reconstitute the behavior of wild-type receptors (Fig. 4). The GluRB and GluRD subunits used for these experiments were edited at the R/G site (28), whereas the GluRA subunits were not, suggesting that this site is not critical for high sensitivity to 2,3-benzodiazepines. To address possible differences in post-translational processing between neurons and oocytes, we assayed receptors generated by poly(A)$^+$ mRNA using RNA isolated from P10 rat forebrain. In control solution, forebrain mRNA-derived receptors showed 58.4 ± 2.8% inhibition by 30 μM GYKI 52466 (Fig. 4A), whereas 30 μM GYKI 53655 produced 94.6 ± 1.3% antagonism (seven experiments) similar to results obtained for native AMPA receptors in cortical neurons (15). For both drugs, the extent of inhibition was significantly greater than that for recombinant AMPA

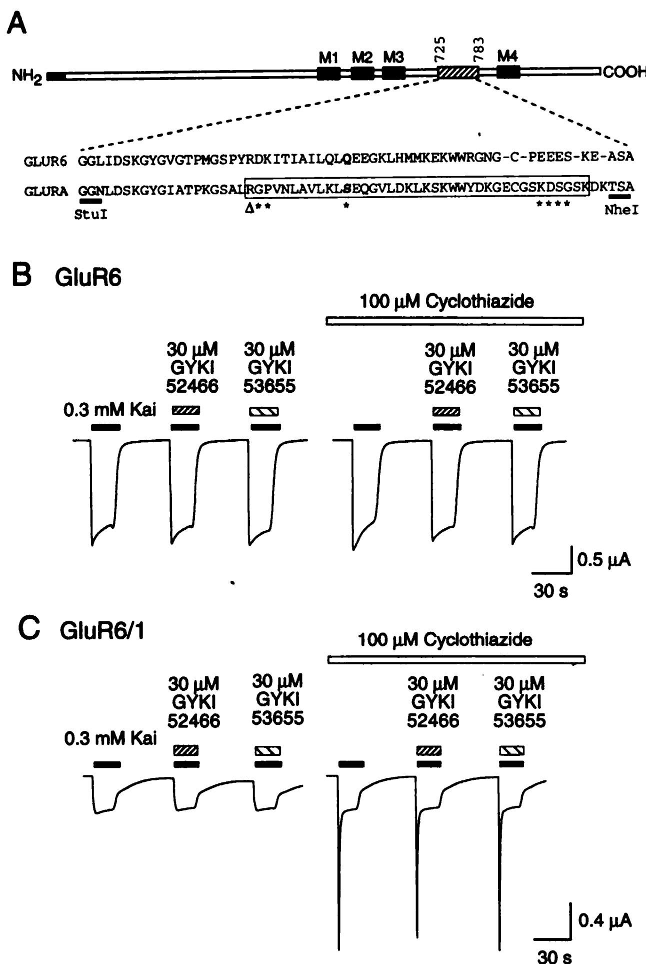

Fig. 3. Insertion of the GluRA flip domain into GluR6 does not confer sensitivity to antagonism by 2,3-benzodiazepines. A, The GluR6/1 chimeric protein is depicted with signal sequence and membrane regions M1 to M4 (*black boxes*) and the region exchanged between GluRA and GluR6 (*striped box*). The unique restriction sites introduced into GluR6, between which residues from GluRA were introduced, are *underlined* in the amino acid sequences shown for GluR6 and GluRA; the flip exon of GluRA is *boxed*; *asterisks*, amino acids that differ between flip and flop splice variants; the site of RNA editing in GluRB, C, and D is marked (Δ); *bold*, the site (GluR6[Q_{755}S]) that confers sensitivity to cyclothiazide in a GluR6 point mutant. B, Responses of wild-type GluR6 evoked by 300 μM kainate after preincubation in 0.3 mg/ml concanavalin A. No antagonism was seen during application of 30 μM GYKI 52466 or GYKI 53655, either in control solution or in the presence of 100 μM cyclothiazide; note that cyclothiazide failed to potentiate responses to kainate. C, Responses to kainate of the chimeric receptor GluR6/1 after preincubation in 0.3 mg/ml concanavalin A are also insensitive to antagonism by GYKI 52466 or GYKI 53655 but showed strong potentiation by 100 μM cyclothiazide; 2,3-benzodiazepines also fail to alter potentiation by cyclothiazide despite introduction of the GluRA flip domain.

receptors ($p < 0.01$). This provides evidence that *Xenopus* oocytes are competent for synthesis of receptors with high sensitivity to 2,3-benzodiazepines. We repeated this experiment using adult hippocampal poly(A)$^+$ mRNA and found that kainate responses were inhibited 63.3 ± 0.5% by 30 μM GYKI 52466 and 97.3 ± 0.2% by 30 μM GYKI 53655 (five experiments). Therefore, potent antagonism by 2,3-benzodiazepines appears to be a general property of receptors encoded by poly(A)$^+$ mRNA, independent of the age of the animal or the region of the brain from which the mRNA is derived.

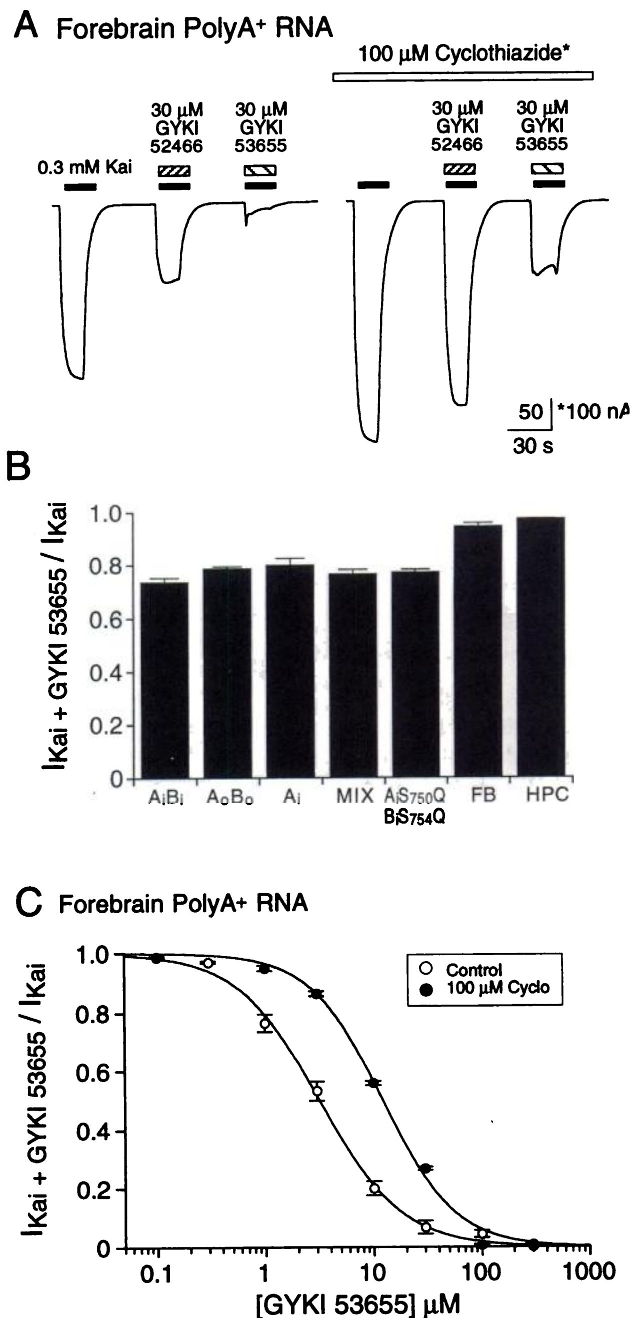

Fig. 4. Kainate responses from receptors generated from rat central nervous system poly(A)$^+$ mRNA are more potently antagonized by 2,3-benzodiazepines than responses from recombinant AMPA receptors. A, Responses evoked by 300 μM kainate from an oocyte that was injected with poly(A)$^+$ mRNA isolated from a P10 rat forebrain. Both GYKI 52466 and GYKI 53655 produce strong inhibition in control solution, with nearly complete block by 30 μM GYKI 52466, which is similar to results obtained for cortical neurons (15). Antagonism by both drugs is less potent in the presence of 100 μM cyclothiazide. Note that the gain for responses evoked in control solution is twice that for responses in the presence of cyclothiazide. B, Inhibition by 30 μM GYKI 53655 for responses recorded from recombinant AMPA receptors and from native AMPA receptors generated from poly(A)$^+$ mRNA isolated from P10 rat forebrain (*FB*) and adult rat hippocampus (*HPC*); *MIX*, combination of plasmids encoding $GluRA_i/A_o/B_i/B_o/D_o$ injected at a ratio of 1:1:5:5:1. C, Dose-inhibition analysis of kainate responses antagonized by GYKI 53655 for oocytes expressing P10 rat forebrain poly(A)$^+$ mRNA, with IC_{50} of 3.0 μM for control (○) and 12.4 μM in the presence of 100 μM cyclothiazide (●, seven experiments); data points are mean ± standard error.

Similar to the behavior of both recombinant AMPA receptors expressed in *Xenopus* oocytes and native AMPA receptors present in cortical neurons, receptors generated by forebrain mRNA showed weaker antagonism by 2,3-benzodiazepines in the presence of 100 μM cyclothiazide (18.1 $\pm$ 1.3% inhibition by 30 μM GYKI 52466 and 72.1 $\pm$ 1.9% inhibition by GYKI 53655). Dose-response curves for GYKI 53655 inhibition of receptors generated by forebrain poly(A)$^+$ mRNA (Fig. 4) confirm this behavior. In control solution, the IC_{50} for GYKI 53655 was 3.2 $\pm$ 0.4 μM. In the presence of 100 μM cyclothiazide, there was a 4-fold rightward shift of the dose-response curve (IC_{50} = 12.4 $\pm$ 0.3 μM).

Discussion

Our experiments demonstrate that a mutation that completely prevents AMPA receptor modulation by cyclothiazide has no effect on modulation by 2,3-benzodiazepines. A chimeric receptor constructed by exchanging an AMPA receptor subunit flip exon with a homologous region in the kainate receptor GluR6 introduces sensitivity to cyclothiazide but does not confer sensitivity to 2,3-benzodiazepines. Based on this, we conclude that the molecular determinants directing allosteric modulation by cyclothiazide are not identical to those directing allosteric modulation by noncompetitive AMPA receptor antagonists. We observed only modest splice variant-dependent differential modulation by 2,3-benzodiazepines, further suggesting that the flip/flop domain does not serve to direct 2,3-benzodiazepine interaction with AMPA receptors. The hypothesis that GYKI 52466 acts to promote desensitization (14) is consistent with the observation that for wild-type AMPA receptors dose-response curves for 2,3-benzodiazepines are shifted rightward in the presence of 100 μM cyclothiazide. However, a critical test of this hypothesis proved inconclusive, in that the IC_{50} for inhibition by GYKI 53655 of the flip variant of AMPA receptors generated from $GluRA_iB_i$ was not significantly different from that for the corresponding flop variant generated by $GluRA_oB_o$, even though these receptors differ considerably in their desensitization kinetics (12, 21, 24, 25). For all benzothiadiazide-sensitive receptors tested in the present experiments, the potency of 2,3-benzodiazepines decreased in the presence of cyclothiazide. Because cyclothiazide has no effect on benzothiadiazide-insensitive receptors generated from the mutants $GluRA_i[S_{750}Q]GluRB_i[S_{754}Q]$, the pseudocompetitive interaction between 2,3-benzodiazepines and cyclothiazide is unlikely to be due to competition at a common binding site and instead most likely reflects allosteric interactions.

Together with prior experiments on the action of antagonists at AMPA receptors with mutations in the agonist binding site (29), our results suggest that the 2,3-benzodiazepine recognition site is unlikely to be in the flip/flop domain or in the agonist binding pocket. Although 2,3-benzodiazepines are selective for AMPA versus kainate receptors, high concentrations of these drugs can produce weak block of kainate receptor responses (15). The extent of amino acid homology between the AMPA and kainate receptor subunit families is only ~40% (1), and therefore it is not possible to make predictions as to the location of the 2,3-benzodiazepine binding site based on sequence comparisons alone. Further analysis using mutagenesis and receptor chimeras will be necessary to map the 2,3-benzodiazepine binding site.

The observation that recombinant AMPA receptors were not antagonized as potently by GYKI 53655 as are native AMPA receptors (15, 17) or AMPA receptors generated by poly(A)$^+$ mRNA is potentially of considerable interest. Our experiments demonstrate that the *Xenopus* oocyte system is competent for synthesis of AMPA receptors that are strongly antagonized by 2,3-benzodiazepines but only when these are translated from poly(A)$^+$ mRNA. This could be explained by (i) a strict requirement for a subunit stoichiometry that was not adequately preserved in any of our coinjection experiments using mixtures of cDNAs for recombinant AMPA receptors, (ii) a post-translational modification that was facilitated by cotranslation with poly(A)$^+$ mRNA, which may supply additional quantities of post-translational modifying enzymes required to enhance antagonism by 2,3-benzodiazepines, and (iii) the requirement for an additional subunit or accessory protein that was supplied by the poly(A)$^+$ mRNA; this could be GluRC, combinations of which were not tested, or an unknown subunit. Differential phenotypes have been seen for other ion channel proteins, such as rat brain sodium channels, when these are expressed in *Xenopus* oocytes using poly(A)$^+$ mRNA versus recombinant RNA. In the case of sodium channels, *in vivo*-like inactivation kinetics could be restored in oocytes when cRNA encoding sodium channel α subunits was coinjected with low molecular weight rat brain RNA, which was subsequently shown to encode a modulatory β subunit (30, 31). Further research will be required to determine whether *in vivo* AMPA receptors also function in conjunction with unidentified modulatory proteins.

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Note Added in Proof

While this paper was in press, a study on wild-type recombinant AMPA receptors was published in which the authors concluded that cyclothiazide and 2,3-benzodiazepines do not compete for the same binding site (32).

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