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Interactions among GYKI-52466, Cyclothiazide, and Aniracetam at Recombinant AMPA and Kainate Receptors

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SUMMARY

We examined the actions of cyclothiazide, aniracetam, and 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI-52466) on recombinant α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors. Receptors expressed in *Xenopus* oocytes or human embryonic kidney 293 cells were characterized using voltage and patch-clamp electrophysiology. Aniracetam and cyclothiazide potentiated AMPA receptor currents by slowing or blocking desensitization. Cyclothiazide was more potent at receptors consisting of flip subunits compared with receptors consisting of flop subunits, whereas aniracetam appeared to be more efficacious at flop receptors. The potency of GYKI-52466 did not differ in heteromeric flip or flop containing AMPA receptors, but GYKI-52466 was less potent at homomeric $GluRA_i$ and $GluRD_i$ receptors. At heteromeric AMPA receptors, 50 μM cyclothiazide increased the IC_{50} value for GYKI-52466 significantly. The increase was largest in $GluRB_i/D_i$ receptors where the IC_{50} value shifted from 21.9 μM (95% confidence interval, 12.0–39.8 μM) to 126 μM (95% confidence interval, 72.4–214 μM) in the presence of cyclothiazide. In contrast, 100 μM GYKI-52466 did not alter the EC_{50} of cyclothiazide at $GluRB_i/D_i$ receptors nor did it markedly change the maximal potentiation induced by cyclothiazide. At $GluRB_i/D_i$ receptors transiently expressed in human embryonic kidney 293 cells, 30 μM GYKI-52466 inhibited the steady state and the peak current evoked by 300 μm L-glutamate to the same extent (34.5 ± 12% and 27.3 ± 13.0%, respectively; five experiments), and GYKI-52466 did not alter the apparent rate of desensitization (τ = 15.7 ± 4.7 and 17.5 ± 8.3 msec in the absence and presence of GYKI-52466, respectively; five experiments). GYKI-52466 inhibited L-glutamate currents in the presence and absence of 10 μM cyclothiazide, but GYKI-52466 never restored the desensitization that was blocked by cyclothiazide. Furthermore, GYKI-52466 inhibited L-glutamate currents mediated by homomeric Glu_6 receptors, which are not potentiated by cyclothiazide. Our data suggest that the effect of cyclothiazide on the affinity of GYKI-52466 for its binding site is allosteric and that the positive modulatory effect of cyclothiazide and the negative modulatory effect of GYKI-52466 result from binding to separate sites on recombinant subunits.

Drugs that modulate neuronal glutamate receptor activity have attracted a great deal of attention because of their potential for enhancing memory and treating certain pathologies. Modulation of the NMDA receptor subtype by compounds such as glycine, Mg^{2+}, Zn^{2+}, and polyamines plays a central role in the physiology of this receptor (for a review, see Ref. 1). Recently, allosteric modulation of AMPA receptor function has also been described. The modulators include compounds that enhance (2–4) and compounds that inhibit (5, 6) AMPA receptor activity. Some of these new AMPA receptor modulators already have shown potential clinical uses. The noncompetitive antagonist GYKI-52466 may be neuroprotective in animal models of cerebral ischemia (7), and 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine, a compound that enhances synaptic transmission mediated by AMPA receptors, is effective at improving memory in experimental animals (8).

The potentiating effects of cyclothiazide and aniracetam appear to involve inhibition or slowing of AMPA receptor desensitization normally elicited by agonists (9, 10). In addition to preventing or slowing desensitization, cyclothiazide slows the decay of currents on removal of agonist, increases the burst duration of single channel currents, and exerts a slight blocking effect on currents (2, 3, 9, 10). The complete spectrum of activity shown by these compounds in neuronal preparations is largely unexplored but may include actions related to cyclothiazide's diuretic effects in peripheral tissues. It is presumed that cyclothiazide and aniracetam share a common mechanism of action, but this has not been rigorously tested.

Negative allosteric modulators of AMPA receptors are ex-

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ABBREVIATIONS: GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; NMDA, *N*-methyl-D-aspartate; GYKI-53655, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney.

emplified by a series of 2,3-benzodiazepines of which GYKI-52466 and GYKI-53655 have been most extensively studied. GYKI-52466 exerts a noncompetitive antagonism of currents mediated by AMPA receptors in cultured hippocampal neurons that is independent of voltage and receptor activation (6), suggesting that GYKI-52466 does not block ion flow through the channel. These compounds block neuronal AMPA receptors more potently than they inhibit currents mediated by kainate receptors (11, 12) and they have no effect on NMDA receptors (5).

To test whether GYKI-52466 blocked receptors by altering desensitization, Zorumski *et al.* (6) examined how cyclothiazide and GYKI-52466 interacted. They showed that GYKI-52466 was less potent in the presence of 10 μM cyclothiazide. Others subsequently reported similar observations *in vivo* using aniracetam and GYKI-52466 (13) and *in vitro* using cyclothiazide and GYKI-53655 (14). These observations led to speculation that the negative modulators, GYKI-52466 and related compounds, and the positive modulators, cyclothiazide and aniracetam, exert their modulatory effect by reciprocal interaction with a common binding site. However, others have observed no pharmacological interactions between cyclothiazide and GYKI-53655 (15, 16), suggesting distinct binding sites for positive and negative modulators. Because neurons express multiple AMPA receptors, it is possible that different subtype selectivity profiles of the 2,3-benzodiazepines cyclothiazide and aniracetam can influence the amount of interaction observed among the compounds.

The AMPA receptor subunits (GluRA through GluRD) exist in the two variants, flip ($_i$) and flop ($_o$), generated by alternative splicing of exons encoding an area near the carboxyl-terminal end of the subunit (17). The flip/flop module has a significant effect on the recovery from and the rate of AMPA receptor desensitization in some recombinant receptor types (18), and cyclothiazide has higher affinity for potentiation of kainate responses at $GluRA_i$ than at $GluRA_o$ (19). These observations suggest that positive and negative modulation may depend on AMPA receptor subunit combination. In an effort to reveal subtype selective actions of modulators and to clarify discrepancies arising from work on neuronal systems, we examined the actions of cyclothiazide, aniracetam, and GYKI-52466 on recombinant AMPA and kainate receptors. We expressed receptors in *Xenopus* oocytes or HEK 293 cells, estimated the affinity of modulators, and studied the pharmacological interactions between pairs of these compounds to determine whether they share mechanisms of action or binding sites on recombinant AMPA receptors. Recombinant expression allows the study of individual receptor subtypes in isolation and thereby eliminates uncertainties due to simultaneous actions of drugs at different receptor subtypes and at sites not directly associated with the receptor. The results suggest that some of the properties and interactions of GYKI-52466, cyclothiazide, and aniracetam depend on subunit combination. Aniracetam and cyclothiazide may share common mechanisms and binding sites, whereas GYKI-52466 probably binds to the receptor in a different location.

Experimental Procedures

Materials. GYKI-52466 was a gift from Dr. I. Taranawa at the Institute for Drug Research, Budapest, Hungary; cyclothiazide was a gift from Eli Lilly; and AMPA was a gift from Parke-Davis Research Laboratories. All other reagents were purchased from regular commercial sources and were of the purest grade available. Subunits were expressed from plasmids carrying cDNA clones downstream of a human cytomegalovirus promoter.

***Xenopus* oocyte injection and electrophysiology.** Ovaries from mature *Xenopus laevis* were removed, and the oocytes were separated and defollicated by collagenase treatment (type II, 1.5 mg/ml) in calcium-free modified Barth's saline (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.5) for 2 hr at room temperature. Stage VI oocytes were isolated, and 1 ng cDNA was injected into the nucleus using a Picospritzer II. For expression of heteromeric combinations, a 1:5 ratio of GluRA or GluRD to GluRB cDNA was injected. The excess GluRB was necessary to prevent the appearance of homomeric GluRA or GluRD in these receptors. After injection, the oocytes were incubated in 96-well tissue culture plates in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM pyruvic acid, pH 7.5) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). The incubation media was changed every 48 hr, and the oocytes were used for voltage-clamp experiments 2–6 days after the injection. Two-electrode voltage-clamp (Axoclamp-2A; Axon Instruments) was used to measure agonist-evoked currents in oocytes at a holding potential of −60 mV. During the experiment, the oocytes were continuously perfused with frog Ringers' solution containing Ba^{2+} instead of Ca^{2+} (100 mM NaCl, 1 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES, pH 7.3) to minimize currents through endogenous Ca^{2+}-activated Cl^- channels (20). Agonists (L-glutamate, kainate) were dissolved directly in the Ba^{2+}-containing frog Ringers' solution, and modulators (cyclothiazide, aniracetam, and GYKI-52466) were dissolved in the Ba^{2+}-containing frog Ringers' solution from prepared stock solutions (30, 500, and 10 mM) in DMSO. The maximal final DMSO concentration never exceeded 2%, a concentration that did not affect the cells. Agonists and modulators were applied by local superfusion of the oocyte (~1 ml/min). Modulators were applied to the oocytes for 1 min to allow for complete equilibration with the receptors before challenge with agonist. Agonists were usually applied for ~30 sec. Currents were filtered at 20 Hz (~3 dB) before being digitized at 100 Hz on-line using an Instrutech ITC-16 analog/digital converter connected to a Macintosh IIci computer.

Transfection and whole-cell recordings of HEK 293 cells. HEK 293 cells were grown in minimum essential medium with Earles's salts, 2 mM glutamine, and 10% fetal calf serum at 37° in a 5% CO_2 humidified incubator. The cells were plated at low density on 35 × 10 mm-tissue culture dishes. At 24–48 hr after plating, the cells were transfected by the $CaPO_4$ precipitation method as described by Chen and Okayama (21), with cDNA encoding the appropriate subunits. A 1:1 ratio of plasmids were transfected to obtain heteromeric receptors containing $GluRA_i/B_i$, $GluRA_o/B_o$, and $GluRB_i/D_i$. The cells were used for patch-clamp electrophysiology 48 hr after the transfection.

Whole-cell patch-clamp recordings were made from isolated cells voltage-clamped at −60 mV. For patch-clamp recording, the cells were continuously perfused with normal rat Ringers' containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. The pipette solution consisted of 140 mM CsCl, 1.0 mM $MgCl_2$, 11 mM EGTA, and 10 mM HEPES, pH 7.3. Modulators (cyclothiazide, aniracetam, and GYKI-52466) were dissolved in normal rat Ringers' from prepared DMSO stock solutions (see above). L-Glutamate was dissolved directly in rat Ringers' and rapidly applied to the cells using a piezo-driven stepper apparatus (22). Currents were filtered at 2000 Hz before being digitized on-line at a frequency of 5000 Hz using a Instrutech ITC-16 interface and a Macintosh-IIfx computer.

Data analysis. The limited solubility of GYKI-52466 and cyclothiazide in physiological saline solutions sometimes precluded the construction of complete concentration-response relations. GYKI-

52466 came out of solution at concentrations >100 μM, and cyclothiazide was not soluble at >150 μM. Thus, proper and consistent estimation of the cyclothiazide EC_{50} and the GYKI-52466 IC_{50} required minor modifications of the procedures usually used to estimate concentration-response curve parameters. The cyclothiazide EC_{50} was estimated by measuring the amplitude of steady state currents evoked by 300 μM L-glutamate in the continuous presence of various concentrations of cyclothiazide and fitting the data from individual oocytes to this equation:

$$I=\{I_{max}/[1+([\text{cyclothiazide}]/EC_{50})^{n_H}]\}+I_{control} \quad (1)$$

where I_{max} represents the maximum steady state currents obtained, I is the steady-state current produced by the agonist, $I_{control}$ is the current evoked by L-glutamate in the absence of cyclothiazide, and n_H is the Hill coefficient. When receptors consisting of all flop subunits were tested, the potentiating effect of cyclothiazide never saturated. The EC_{50} estimates that appear in Table 1 represent the concentration of cyclothiazide necessary to produce half of the potentiation caused by 150 μM cyclothiazide. These values, therefore, represent a minimum estimate of the EC_{50}. To estimate the potency (IC_{50}) of GYKI-52466, current amplitudes were fitted to this equation:

$$I/I_{max}=1/\{1+[(\text{GYKI-52466})/IC_{50}]\} \quad (2)$$

This equation assumes that the Hill coefficient was equal to 1 and was used because in some situations (especially in the presence of cyclothiazide; Fig. 1), the maximum concentration of GYKI-52466 (100 μM) did not block the currents sufficiently to allow for a proper fit to the logistic equation when the Hill coefficient was allowed to float. To validate this approach, data from concentration-response experiments measured in the absence of cyclothiazide were analyzed with a variant of eq. 1 that included a floating Hill coefficient term, and the values obtained from 24 cells were not significantly different from unity ($p > 0.1$). Moreover, the EC_{50} values obtained from fits that included a floating Hill coefficient were no different from those done using a Hill coefficient fixed at 2. Therefore, fits to eq. 2 allow proper estimation of the IC_{50} for GYKI-52466 block of currents and, more important, facilitate accurate comparisons between the apparent potencies of GYKI-52466 in the presence and absence of cyclothiazide.

TABLE 1

EC_{50} values for cyclothiazide

The EC_{50} values for cyclothiazide were obtained from analysis of 300 μM L-glutamate evoked steady state currents at recombinant AMPA receptors expressed in *Xenopus* oocytes in the presence of 0, 0.3, 1, 3, 10, 30, 100, and 150 μM cyclothiazide. At $gluRA_i/B_i$, $gluRB_i/D_i$, and $gluRD_i$, complete concentration-response curves were obtained (see Fig. 1C) and EC_{50} values were estimated by iterative least-squares curve fit as described in Experimental Procedures. Mean values and 95% confidence intervals were calculated using log of individual EC_{50} values, and *n* is the number of oocytes tested. At $gluRA_o/B_o$ and $gluRB_o/D_o$, the affinity of cyclothiazide was lower and it was not possible to obtain complete concentration-response curves (see Fig. 1C). The EC_{50} value must be equal to or more than the cyclothiazide concentration that potentiates the glutamate current 50% compared with the potentiation obtained using 150 μM cyclothiazide.

	EC_{50}	Hill coefficient	*n*
	μM		
$gluRA_i/B_i$	6.6 (3.4–13)	1.69 ± 0.05	6
$gluRB_i/D_i$	5.0 (4.5–5.5)	1.94 ± 0.07	5
$gluRD_i$	5.9 (4.2–8.5)	1.48 ± 0.08	3
$gluRA_o/B_o$	>76		5
$gluRB_o/D_o$	>91		5

Desensitization rates were determined by fitting the current decays to single exponential equations. All fits were done using an iterative least-squares fitting program (IGOR, Wavemetrics). Mean values and 95% confidence intervals were calculated using the log of individual IC_{50} or EC_{50} estimates. All other values are given as mean ± standard error. Differences between experimental groups were analyzed by two-tailed unpaired Mann-Whitney test.

Results

To estimate the affinity of modulators, receptors were expressed in *Xenopus* oocytes and studied with two-electrode voltage-clamp. These large cells are useful for pharmacological studies at equilibrium, but their size precludes the rapid application of agonists needed to visualize receptor desensitization. Therefore, some studies were done using HEK 293 cells transiently expressing the receptor subunits. Robust agonist currents were observed in each type of cell on expression of the subunit cDNAs, and the properties we measured did not differ between these two host cell systems. No currents were observed in response to any of the modulators alone, nor were currents evoked by receptor agonists seen in uninjected oocytes and untransfected HEK 293 cells. Coexpression of GluRB subunits at the ratios specified in Experimental Procedures resulted in receptors showing linear or outwardly rectifying current-voltage relations (not shown). This indicates that a large majority of the receptors formed on co-expression were heteromeric assemblies. We cannot completely exclude the possibility that some homomeric channels may contribute to the currents measured in these cells, but the relative proportion of homomeric receptors appears to be quite low.

AMPA receptors consisting of only flip subunits ($GluRD_i$, $GluRA_i/B_i$, and $GluRB_i/D_i$) or only flop subunits ($GluRA_o/B_o$ and $GluRB_o/D_o$) were expressed in *Xenopus* oocytes and examined for their sensitivity to cyclothiazide or aniracetam. In oocytes expressing flop receptors, slow desensitization was always observed in the presence of cyclothiazide, whereas in flip receptors, desensitization was seen only occasionally and only with low concentrations (<10 μM) of cyclothiazide (Fig. 1A). Currents observed in the presence of aniracetam did not show marked desensitization at this time scale (Fig. 1B). Steady state currents evoked by 300 μM L-glutamate were measured in the continuous presence of potentiator, and concentration-response relations were constructed (Fig. 1C and Table 1). The potentiation of flip receptors by cyclothiazide showed clear saturation, allowing us to estimate the EC_{50} using eq. 1 (see Experimental Procedures). However, the limited solubility of cyclothiazide and its apparent lower potency at flop receptors precluded accurate estimates of the EC_{50}. Rough estimates of the minimum cyclothiazide EC_{50} (Table 1) in flop receptors suggested that its potency was 11–16 times lower in flop receptors than it was at flip receptors depending on the exact subunits involved. The potentiation induced by high concentrations of cyclothiazide was not different between flip and flop receptors. The aniracetam potentiation also did not saturate within the solubility limits of this compound, so no estimates of potency were attempted. However, the magnitude of the potentiation seemed much greater in flop receptors than it was in flip receptors (Fig. 1C), and aniracetam (5 mM) potentiated the L-glutamate cur-

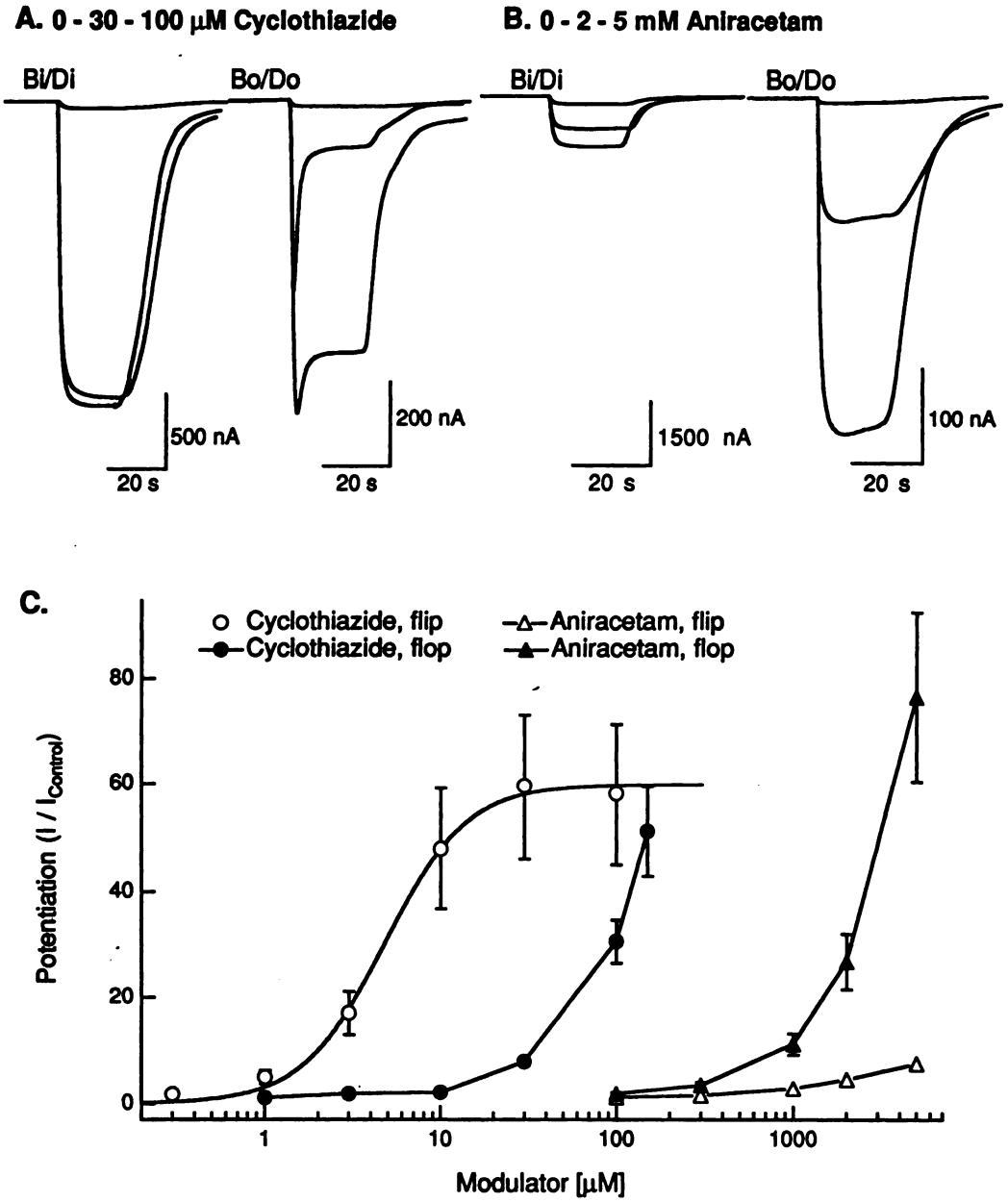

Fig. 1. Cyclothiazide and aniracetam show selectivity for flip versus flop splice variants of AMPA receptors expressed in *Xenopus* oocytes. A and B, Traces showing currents evoked by 300 μM L-glutamate at $GluRB_i/D_i$ and $GluRB_o/D_o$ receptors in the presence of 0, 30, and 100 μM cyclothiazide and in the presence of 0, 2, and 5 mM aniracetam. Current records are presented so that the size of the control currents is the same for each subunit combination. Note the difference in calibration bars. C, Concentration-response relations for cyclothiazide and aniracetam. The points represent mean ± standard error (five experiments) of the potentiation of steady state current at $GluRB_i/D_i$ (*open symbols*) and at $GluRB_o/D_o$ receptors (*closed symbols*). For cyclothiazide's potentiation of currents evoked at $GluRB_iD_i$ receptors where the maximum potentiation could be estimated accurately, the curve represents the least-squares fit to eq. 1 (see Experimental Procedures). The other lines simply connect the points. Currents were evoked by 300 μM L-glutamate and measured at a holding potential of −60 mV. Because the amount of receptor expression varies between oocytes, all data have been normalized by dividing the current measured in the presence of different cyclothiazide concentrations with the current measured in the absence of cyclothiazide.

rent mediated $GluRA_o/B_o$ receptors 17 ± 3.5-fold versus 8.1 ± 2.2-fold at $GluRA_i/B_i$ receptors and 7.5 ± 1.7-fold at $GluRB_i/D_i$ receptors versus 77 ± 16-fold at $GluRB_o/D_o$ receptors.

We examined the actions of aniracetam and cyclothiazide on desensitization more closely by expressing the receptors in HEK 293 cells and rapidly applying 1 mM L-glutamate. The properties of $GluRA_i/B_i$ receptors were compared with those of receptors constructed of $GluRA_o/B_o$ subunits. We used 30 μM cyclothiazide and 1 mM aniracetam because these concentrations potentiated steady state currents to a similar degree in oocytes expressing only flop subunits. Similar to its effects in oocytes (Table 1 and Fig. 1C), 30 μM cyclothiazide produced near-maximal potentiation at flip receptors but not at flop receptors. The extent of steady state current potentiation could not be compared in HEK 293 cells because flop receptors exhibited no steady state current in the absence of modulators. Fast application of 1 mM L-glutamate evoked rapidly desensitizing currents that decayed to a steady state level, the relative amplitude of which depended on the nature of the splice variant. For $GluRA_i/B_i$ receptors, the average time constant of desensitization was 9.76 ± 0.82 msec (four experiments), and the current desensitized to 8.8 ± 1.4% of the peak current. Aniracetam slowed the desensitization rate to 15.12 ± 1.18 msec in these cells, increased the amount of nondesensitizing current to 21.5 ± 8.3% of the peak current, and potentiated the peak current amplitude to 1.43 ± 0.05 times that of control. Cyclothiazide increased the current amplitude to 3.96 ± 0.55 times the peak control current and prevented observable desensitization altogether (Fig. 2). In cells expressing $GluRA_o/B_o$, the desensitization rate for currents evoked by 1 mM L-glutamate was 5.82 ± 0.24 msec (six experiments). Aniracetam caused a 2.73 ± 0.22-fold increase in the peak current and reduced the rate of desensitization to 13.3 ± 1.67 msec. After 1 sec, the current desensitized to 9.9 ± 4.6% of the peak current. The amount of potentiation was

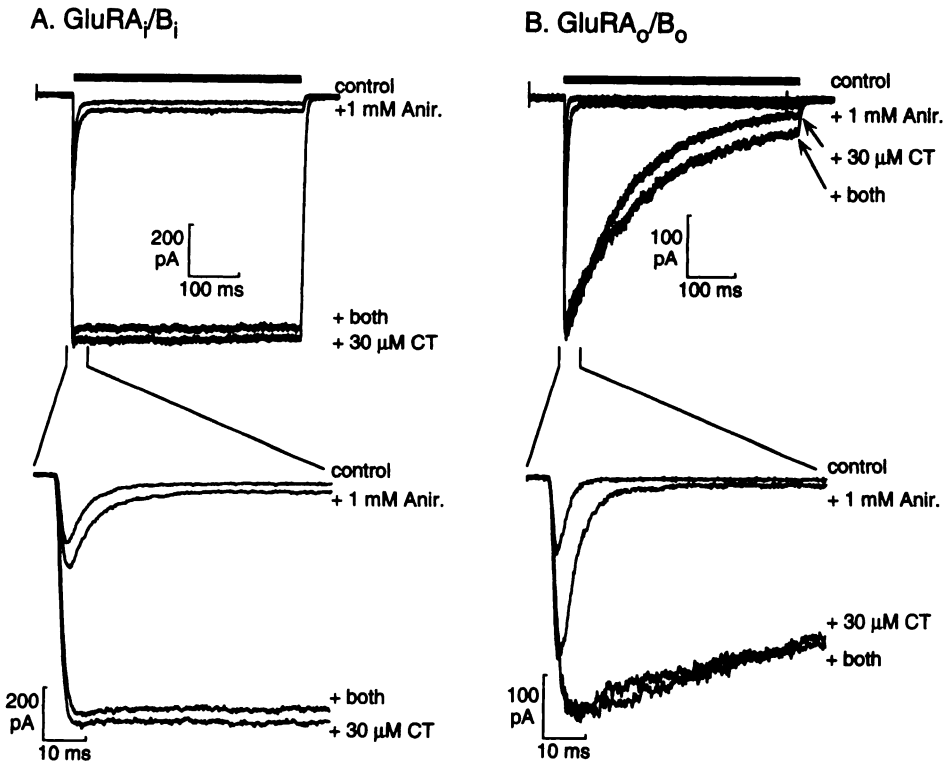

Fig. 2. Potentiation by cyclothiazide (*CT*) and aniracetam (*Anir.*) is not strictly additive. Traces show the effect of 1 mM aniracetam, 30 μM cyclothiazide, and simultaneous application of both on currents evoked by rapid application of 1 mM L-glutamate at GluRA$_i$/B$_i$ (A) and GluRA$_o$/B$_o$ (B) receptors expressed in HEK 293 cells. The holding potential was −60 mV. *Bottom*, initial parts of the traces are expanded to illustrate the initial rapid desensitizing part of the response.

similiar to the potentiation caused by 1 mM aniracetam at GluRA$_o$/B$_o$ receptors expressed in oocytes (4.3 ± 0.5-fold). The peak current evoked by L-glutamate in the presence of cyclothiazide was 2.61 ± 0.10 times that of control. Unlike cells expressing GluRA$_i$/B$_i$, desensitization was slowed by cyclothiazide but not eliminated (average rate in the presence of 30 μM cyclothiazide was 314 ± 14.9 msec, six experiments), and after 1 sec, the current was 4.1 ± 0.9% of the peak current. Thus, although 30 μM cyclothiazide slowed the rate of desensitization to a greater degree in cells expressing only flop subunits, the extent of desensitization was not different from that induced by 1 mM aniracetam.

The pharmacological interactions between cyclothiazide and aniracetam were also tested in this group of HEK 293 cells by applying the modulators together (Fig. 2). For both GluRA$_i$/B$_i$ and GluRA$_o$/B$_o$, the potentiating effects of aniracetam and cyclothiazide on the peak current were not additive. In GluRA$_i$/B$_i$ receptors, no desensitization was observed in the presence of both aniracetam and cyclothiazide (Fig. 2), and the mean current amplitude was slightly lower than it was in the presence of cyclothiazide alone (average amplitude with 30 μM cyclothiazide, 622 ± 298 pA; amplitude with 1 mM aniracetam, 216 ± 129 pA; amplitude with both, 583 ± 279 pA; three experiments). Peak currents mediated by GluRA$_o$/B$_o$ receptors were likewise not additive in the presence of both potentiators (average amplitude with 30 μM cyclothiazide, 301 ± 150 pA; amplitude with 1 mM aniracetam, 256 ± 127; amplitude with both, 319 ± 159 pA; three experiments; Fig. 2), but the addition of 1 mM aniracetam to 30 μM cyclothiazide further slowed the rate of desensitization from 284 ± 2.4 msec in 30 μM cyclothiazide only to 342 ± 13 msec in the presence of both ($p < 0.05$, paired t test, three experiments). The current desensitized to 11 ± 1.0% of the peak current. The slowing of desensitization produced steady state currents that did appear to be additive. The average amplitudes of currents measured after a 1-sec application of L-glutamate were 16.6 ± 8.5 pA (30 μM cyclothiazide), 11.4 ± 6.3 pA (1 mM aniracetam), and 37.0 ± 16.3 pA (both) (three experiments, Fig. 2).

In contrast to cyclothiazide and aniracetam, GYKI-52466 did not differentiate between AMPA receptor splice variants (Table 2). The GYKI-52466 IC$_{50}$ measured in *Xenopus* oocytes was similar at all heteromeric receptors containing GluRB$_i$ or GluRB$_o$ (19.1–30.9 μM), but the affinity for blocking the homomeric GluRA$_i$ and GluRD$_i$ receptors was significantly lower (IC$_{50}$ = 117 μM and 66 μM, respectively; $p <$ 0.05 for any pairwise comparison between GluRA$_i$ or GluRD$_i$ and a GluRB-containing receptor). Because the IC$_{50}$ value for GYKI-52466 at GluRB$_i$/D$_i$ receptors did not depend on the agonist used to activate the receptors (Table 2), 300 μM kainate was used for studies of GYKI-52466 inhibition at GluRA$_o$/B$_o$ and GluRB$_o$/D$_o$ receptors to obtain larger steady state currents. GYKI-52466 inhibition was also examined in HEK 293 cells expressing GluRB$_i$/D$_i$ receptors (Fig. 3). The IC$_{50}$ for GYKI-52466 measured in HEK 293 cells was identical to that determined in *Xenopus* oocytes (IC$_{50}$ for blocking the peak current evoked by 300 μM L-glutamate = 19.1 μM with a 95% confidence interval of 13.8–26.3 μM, five experiments). Neither the extent nor the rate of desensitization was significantly altered by 30 μM GYKI-52466. Peak currents evoked by 300 μM L-glutamate were reduced to 27.3 ± 13% of control by 30 μM GYKI-52466, and the steady state currents were reduced to 34.5 ± 12.0% of control (six experiments). The time constant of desensitization in these six cells was 15.7 ± 4.7 msec in the absence of GYKI-52466 and 17.5 ± 8.3 msec in its presence.

In the presence of 50 μM cyclothiazide, the IC$_{50}$ value for GYKI-52466 measured in oocytes was significantly higher at all the heteromeric receptor combinations ($p < 0.05$) (Table 2). The largest effect was seen at GluRB$_i$/D$_i$ receptors, where

TABLE 2

IC_{50} values for GYKI-52466

IC_{50} values for GYKI-52466 in the absence and in the presence of 50 μM cyclothiazide at different recombinant AMPA receptors expressed in *Xenopus* oocytes. Both agonists were used at 300 μM. The IC_{50} values are the mean values of IC_{50} values from four to six separate experiments (as indicated by *n*) obtained by iterative least-squares curve fit as described in Experimental Procedures. The 95% confidence intervals are shown in parentheses.

Subunit	Agonist	− Cyclothiazide		+ 50 μM Cyclothiazide	
		IC_{50}	*n*	IC_{50}	*n*
		μM		μM	
$GluRA_i/B_i$	L-Glutamate	30.9 (20.4–47.9)	4	100 (67.6–148)	4
$GluRB_i/D_i$	L-Glutamate	21.9 (12.0–39.8)	5	126 (72.4–214)	5
$GluRB_i/D_i$	Kainate	30.2 (24.0–38.0)	5	93.3 (79.4–110)	4
$GluRA_i$	L-Glutamate	117 (81.3–174)	5	166 (70.8–380)	5
$GluRD_i$	L-Glutamate	66.1 (50.1–85.1)	5	83.2 (51.3–135)	6
$GluRA_o/B_o$	Kainate	19.1 (11.2–31.6)	5	29.5 (25.7–34.7)	5
$GluRB_o/D_o$	Kainate	21.9 (17.8–27.5)	5	32.4 (29.5–35.5)	5

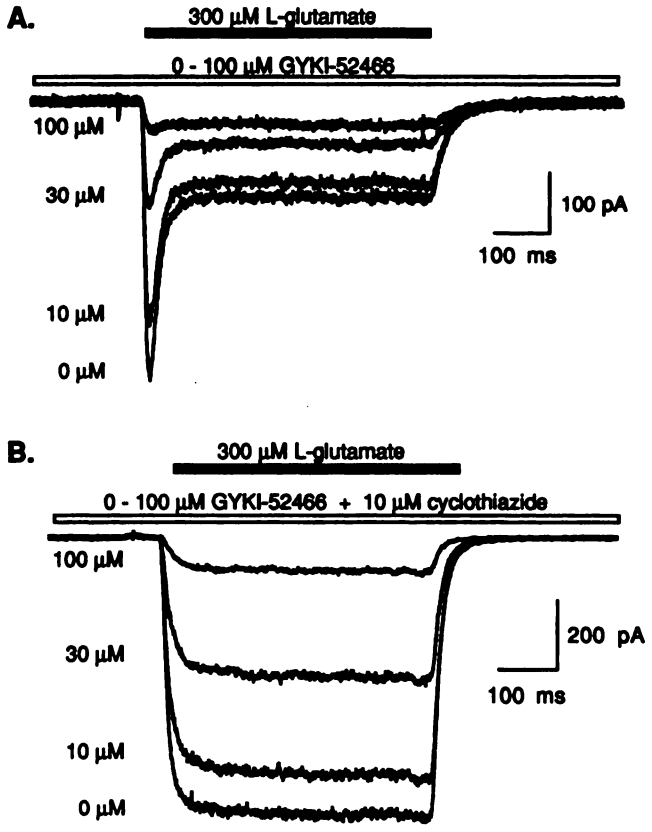

Fig. 3. GYKI-52466 does not reverse the block of desensitization caused by cyclothiazide. GYKI-52466 inhibition of L-glutamate (300 μM) evoked currents at $GluRB_i/D_i$ receptors expressed in HEK 293 cells in the absence (A) or presence (B) of 10 μM cyclothiazide. GYKI-52466 inhibited currents both in the presence and absence of cyclothiazide but did not restore desensitization to cyclothiazide-potentiated receptors.

the IC_{50} value changed from 21.9 to 126 μM (Fig. 4 and Table 2). The GYKI-52466 inhibition curve was shifted by 50 μM cyclothiazide to a greater degree at $GluRA_i/B_i$ and $GluRB_i/D_i$ receptors than at the corresponding flop receptors (Table 2). At $GluRA_i/B_i$ and $GluRB_i/D_i$, the IC_{50} values were increased 3.2- and 5.8-fold compared with a 1.5-fold increase at $GluRA_o/B_o$ and $GluRB_o/D_o$ receptors. The smaller shift seen in flop receptors corresponded to the lower potency of cyclothiazide for potentiating currents mediated by flop receptors. At homomeric $GluRA_i$ and $GluRD_i$ receptors, the GYKI-52466 IC_{50} was not significantly different in the presence of 50 μM cyclothiazide ($p = 0.15$ and 0.25, respectively), probably because of their lower sensitivity to GYKI-52466 inhibition in the absence of cyclothiazide (Fig. 4 and Table 2). When desensitization of $GluRB_i/D_i$ receptors was examined in HEK 293 cells, currents induced by 300 μM L-glutamate showed no obvious desensitization in the presence of 10 μM cyclothiazide. GYKI-52466 blocked the currents in the presence of cyclothiazide, but desensitization was not observed even at high (100 μM) concentrations of GYKI-52466 (Fig. 4). Although GYKI-52466 showed a clear blocking effect in these experiments, it did not reverse the effects of cyclothiazide on receptor desensitization.

Changes in the GYKI-52466 IC_{50} caused by cyclothiazide suggest that these compounds may compete for the same binding site on AMPA receptors. Alternatively, the change in apparent affinity may result indirectly from conformational changes caused by cyclothiazide. To clarify the nature of the cyclothiazide/GYKI-52466 interaction, we measured the cyclothiazide EC_{50} for potentiating currents in the presence of GYKI-52466. The $GluRB_i/D_i$ subunit combination was chosen for this experiment because cyclothiazide shifted the GYKI-52466 inhibition curve to the largest extent in this receptor type. Fig. 5 shows that GYKI-52466 has little effect on the cyclothiazide concentration-response curve. The average cyclothiazide EC_{50} in the presence of 100 μM GYKI-52466 was 4.2 μM (95% confidence interval, 3.2–5.5 μM) compared with an EC_{50} of 5.0 μM (95% confidence interval, 4.5–5.5 μM) in the absence of antagonist. Comparison of the maximal potentiation in the absence and presence of 100 μM GYKI-52466 in five oocytes showed that cyclothiazide overcomes the inhibitory effect of GYKI-52466 (Fig. 5B). The ability of cyclothiazide to increase the steady state current amplitude was only slightly reduced (estimated maximum potentiation by cyclothiazide was 90.8 ± 3.8% of control in the presence of 100 μM GYKI-52466) even though currents were blocked by 100 μM GYKI-52466 to 18 ± 5.4% control in the absence of cyclothiazide.

Experiments were also carried out to assess the selectivity of the modulators for AMPA versus kainate receptors. For this study, we expressed GluR6 in HEK 293 cells and measured the currents on rapid application of L-glutamate (Fig. 6). We chose the HEK 293 cells for these studies because the rapid and profound desensitization exhibited by GluR6 receptors inhibits our ability to measure such currents in *Xenopus* oocytes. Neither cyclothiazide (10 μM) nor aniracetam (2 mM) altered currents mediated by GluR6 receptors (Fig. 6).

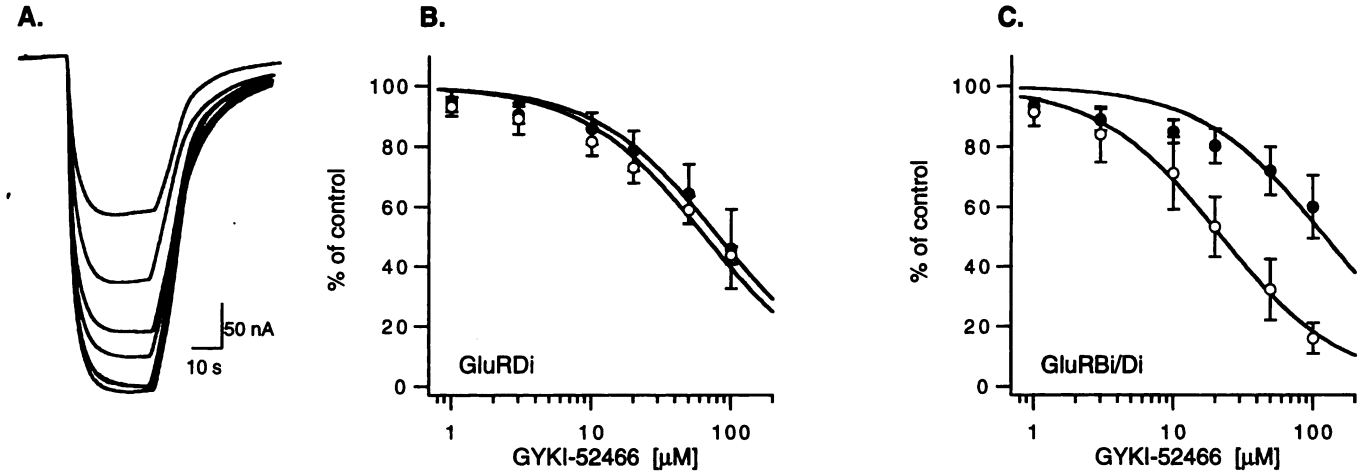

Fig. 4. GYKI-52466 inhibition of 300 μM L-glutamate evoked currents at $GluRD_i$ and $GluRB_i/D_i$ receptors expressed in *Xenopus* oocytes after cDNA injections. A, An example of traces obtained at $GluRD_i$ receptors in the presence of 0, 1, 3, 10, 20, 50, and 100 μM GYKI-52466. B and C, GYKI-52466 inhibition curves in the absence (○) and presence (●) of 50 μM cyclothiazide. *Points in the graph* represent the amplitude of steady state currents evoked by 300 μM glutamate in the presence of different GYKI-52466 concentrations as percentage of maximum response in absence of antagonist (mean ± standard deviation of five or six experiments). *Curves* represent least-squares fit of the points to eq. 2 (see Experimental Procedures).

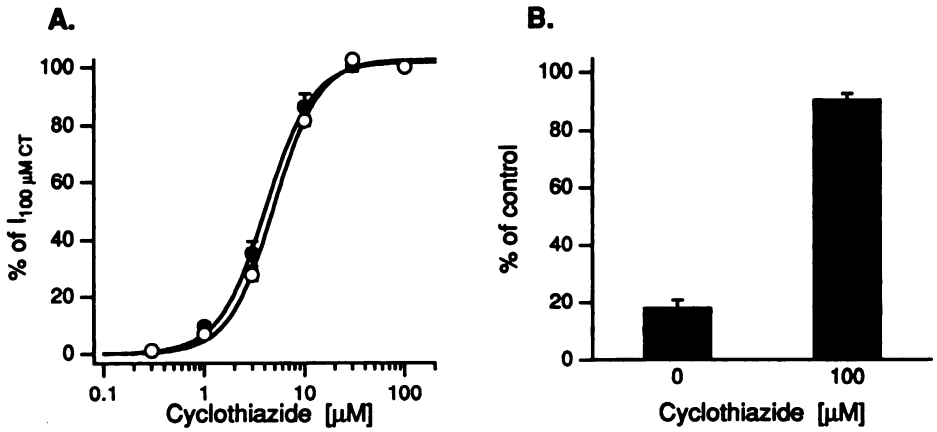

Fig. 5. GYKI-52466 does not change the apparent affinity of cyclothiazide for $GluRB_i/D_i$ receptors expressed in *Xenopus* oocytes. A, Shown are the concentration-response relations for cyclothiazide potentiation in the absence (○) and presence (●) of 100 μM GYKI-52466. *Symbols* represent the mean ± standard error for five cells. *Lines* represent fits of these data to eq. 1 (see Experimental Procedures). The data have been normalized according to the maximal potentiation evoked by cyclothiazide in the absence of GYKI-52466. B, The blocking effect of 100 μM GYKI-52466 was completely overcome by 100 μM cyclothiazide. *Bars* represent the mean ± standard error (five experiments) percentage of control current not blocked by 100 μM GYKI-52466 in the absence or presence of 100 μM cyclothiazide. Control currents were measured in the presence of the indicated concentration of cyclothiazide.

However, GYKI-52466 did show some blocking actions at these kainate receptors. The potency of GYKI-52466 was markedly lower at GluR6 receptors than it was at the AMPA receptor types. The average IC_{50} was 112 μM (95% confidence interval, 65–195 μM, four experiments).

Discussion

The results presented above show that cyclothiazide has a greater apparent affinity for receptors made of subunits carrying the flip splice variants than for those carrying flop subunits. The potency of aniracetam apparently does not differ markedly between flip and flop AMPA receptors, but the magnitude of steady state current potentiation by 5 mM aniracetam is greater for flop receptors than for flip receptors. The actions of these two potentiators on $GluRA_i/B_i$ are not additive, suggesting that they share at least a common mechanism of action. The noncompetitive AMPA receptor blocker GYKI-52466 appears to be somewhat more potent at blocking heteromeric AMPA receptors containing a GluRB subunit than it is at homomeric AMPA receptors made of $GluRA_i$ or $GluRD_i$ or at kainate receptors consisting of GluR6. Cyclothiazide shifts the GYKI-52466 inhibition curve to the right in heteromeric AMPA receptors, but GYKI-52466 does not reverse the block of desensitization induced by cyclothiazide or alter its potency. Cyclothiazide does not shift the GYKI-52466 inhibition curve in homomeric $GluRA_i$ or $GluRD_i$ receptors, and neither cyclothiazide nor aniracetam shows potentiating effects on kainate receptors made of GluR6.

Previous studies have identified differences in cyclothiazide sensitivity between flip and flop AMPA receptors (19). In these studies, cyclothiazide showed a markedly lower potency for flop receptors when kainate was used to activate the receptors. The present results show that the difference in

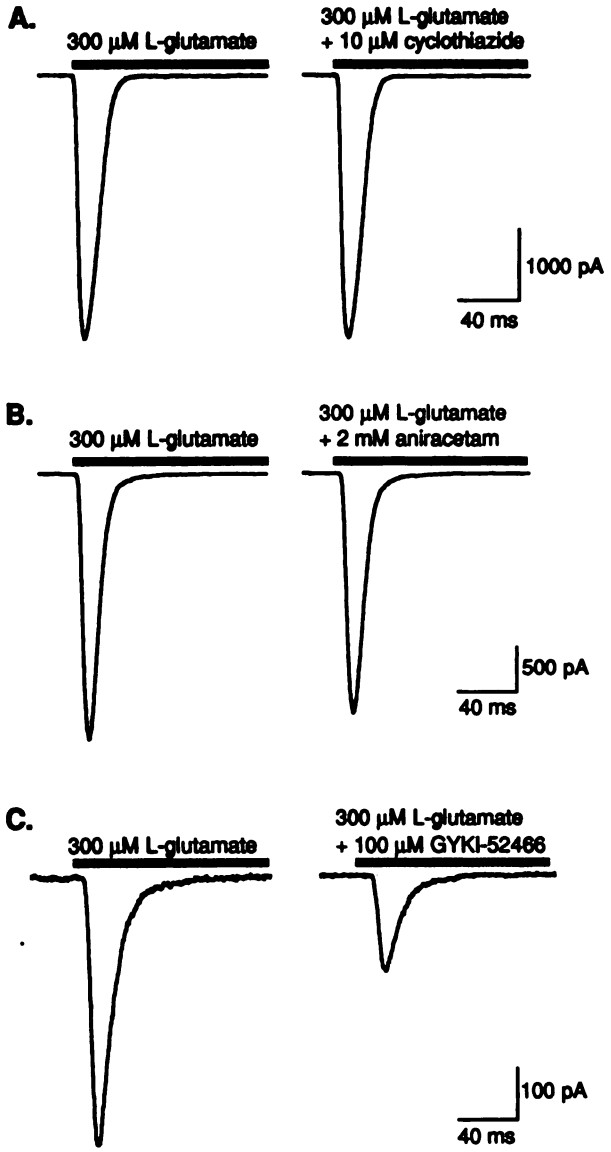

Fig. 6. Positive and negative modulators show a clear pharmacological separation at recombinant kainate receptors. Traces showing the effect on currents evoked by rapid application of 300 μM L-glutamate at HEK 293 cells expressing GluR6 receptors by 10 μM cyclothiazide (A), 2 mM aniracetam (B), and 100 μM GYKI-52466 (C). The application of L-glutamate continued longer than the traces shown.

cyclothiazide potency is likewise apparent when L-glutamate is used as agonist, but there appears to be no difference between flip and flop receptors in the maximum potentiation of L-glutamate currents. Because the effect of cyclothiazide never saturated in flop receptors, it is possible that even greater potentiation would be seen if higher concentrations of cyclothiazide could be tested. Aniracetam also potentiates flop receptors more than flip receptors, even though its potency does not appear to differ between flip and flop receptors. The magnitude of steady state current potentiation probably reflects the intrinsic rate and extent of desensitization as well as the binding and unbinding kinetics of the modulators. Using ultrafast agonist application, Mosbacher *et al.* (18) showed that only the $GluRD_o$ subunit strongly controls the rate of desensitization and that the extent of desensitization does not vary between splice variants. It appears that systematic differences between flip and flop receptors are seen only when measuring the kainate/L-glutamate steady state current ratios (17) and the potency of cyclothiazide (19).

When cyclothiazide and aniracetam are compared, there appears to be a large difference in the affinity of these compounds regardless of the splice variant, but 5 mM aniracetam has at least the same the relative efficacy as cyclothiazide at $GluRB_o/D_o$ receptors. However, 5 mM aniracetam is markedly less effective at potentiating flip receptors than is cyclothiazide. Because of aniracetam's limited solubility, we were unable to determine whether differences in its effect on flip and flop receptors were due to differences in affinity or alterations in efficacy. The rate of desensitization is always slowed to a greater extent by 30 μM cyclothiazide compared with 1 mM aniracetam, but the degree of desensitization does not differ. The interactions of aniracetam and cyclothiazide when applied together suggest that they may share a common binding site and mechanism of action. For $GluRA_i/B_i$ receptors current amplitudes are slightly smaller in the presence of both compounds than they are in the presence of 30 μM cyclothiazide alone. This is consistent with aniracetam being a partial agonist and cyclothiazide being a full agonist at the same receptor. The results with $GluRA_o/B_o$ receptors are somewhat more complicated. The potentiation of the peak current does not appear to be additive, and in some cells the peak current in the presence of aniracetam was larger than the peak current seen with cyclothiazide. However, the potentiation of the steady state component did appear to be additive. It is likely that these two compounds also share a common binding site on flop receptors, but the kinetics of their actions appear to be more complex. Further research will be necessary to completely understand the mechanisms of drug action and desensitization. Site-directed mutagenesis studies have shown that differential sensitivity of flip and flop receptors to cyclothiazide is conferred by a single amino acid residue in the alternatively spliced cassette (23). It would be of interest to test the effects of aniracetam on these mutant receptors.

GYKI-52466 inhibited currents mediated by recombinant AMPA receptors in a fashion similar but not identical to that seen in neuronal AMPA receptors. The IC_{50} values reported here are slightly lower than those found in hippocampal neurons (20–30 μM in our cells versus 7–14 μM in neurons) (5, 6). The Hill coefficients observed using recombinant receptors are not different from unity, whereas they are >1 (1.2–1.5) in neuronal preparations. In other respects, the effects of GYKI-52466 are identical to its actions at neuronal AMPA receptors. GYKI-52466 did not alter the rate or extent of desensitization (5), nor was its effect voltage or use dependent (data not shown). The lower potency of GYKI-52466 at GluR6 receptors is consistent with its lower potency at kainate-preferring receptors in native DRG neurons (11). Homomeric $GluRA_i$ or $GluRD_i$ receptors also showed a markedly lower affinity for GYKI-52466 than did the other heteromeric AMPA receptors. This may suggest that GYKI-52466 binds more strongly to the GluRB subunit. However, detailed structure-function analysis will be necessary to identify the amino acid residues and subunits involved in GYKI-52466 binding.

The present data indicate that cyclothiazide and GYKI-52466 have some type of pharmacological interaction but

that this is not due to a direct competition between the two compounds for the same binding site. The reasons for this conclusion are that (a) in the presence of low concentrations of cyclothiazide (10 μM), GYKI-52466 still shows inhibition, but it does not restore desensitization to cyclothiazide-potentiated receptors; (b) GYKI-52466 (100 μM) does not shift the cyclothiazide concentration-response curve for potentiation of the steady state current; (c) cyclothiazide does not shift the GYKI-52466 inhibition curve in homomeric $GluRA_i$ and $GluRD_i$ receptors even though the action of cyclothiazide on these receptors is not markedly different; and (d) kainate receptors are inhibited by GYKI-52466 but are not affected by cyclothiazide (11, 12, 24), suggesting that glutamate receptor structures that bind GYKI-52466 do not necessarily bind cyclothiazide. How, then, does cyclothiazide shift the GYKI-52466 inhibition curve? One possibility is that conformational changes induced by cyclothiazide reduce the affinity of the receptor for GYKI-52466. This hypothesis is supported by the observation that the potentiation by cyclothiazide is decreased only slightly by 100 μM GYKI-52466. An alternative explanation is that there are multiple binding sites for cyclothiazide on the receptor. GYKI-52466 binds to one of those sites, and a different site mediates cyclothiazide's block of desensitization. Partin *et al.* (19) reported that cyclothiazide induces a small inhibitory effect as well as a potentiating action on recombinant AMPA receptors, which is consistent with the latter hypothesis. However, our results with flop receptors in which the ability to potentiate currents appears to correlate with the magnitude of the GYKI-52466 curve shift argue against this explanation. Direct structural studies aimed at mapping the binding sites for these drugs are necessary to resolve this issue.

Studies done in a variety of neuronal preparation have given seemingly contradictory results concerning interactions between GYKI-52466 and cyclothiazide. Aniracetam or cyclothiazide reverses the block of GYKI-52466 (13) or a structural analogue of GYKI-52466, GYKI-53655 (14), in slice preparations and *in vivo*. Moreover, Zorumski *et al.* (6), by measuring kainate-induced currents with patch-clamp, have shown that cyclothiazide and a structurally related compound, hydrocholorothiazide, markedly shift the GYKI-52466 inhibition curve to the right in cultured hippocampal neurons. In contrast, Rammes *et al.* (16) showed that GYKI-52466 applied in the presence of cyclothiazide reduced peak amplitude but could not reverse cyclothiazide-induced prolongation of EPSP. Other studies with different preparations of native receptors show no interaction among cyclothiazide and GYKI-52466 (15, 25). The present results help to reconcile some of these discrepancies because it is now clear that the magnitude of the cyclothiazide-induced shift in GYKI-52466 inhibition varies with subunit combination. Thus, results obtained in neurons derived from different brain regions may give different outcomes depending on the subunits that contribute to the AMPA receptor response.

Better understanding of the molecular nature of the GYKI-52466 and the aniracetam and cyclothiazide binding sites on AMPA receptors is clearly important. GYKI-52466 has been shown to have anticonvulsive actions (26) and neuroprotective effects after experimental ischemia (7). Enhancement of AMPA receptor function by compounds like aniracetam and cyclothiazide appears to improve memory (8). Thus, there is a great deal of therapeutic potential for drugs of both categories. Drugs with selectivity for particular AMPA receptor variants may be especially useful if each receptor subtype subserves different physiological or pathological processes. Data presented here and in previous studies (11, 19) show that subtype selectivity can be attained for positive and negative modulators of AMPA receptors. Further screening of currently available compounds on different recombinant AMPA receptors appears to be justified. Structural information about the binding sites for these compounds will facilitate this effort and lead to better, more selective drugs.

Acknowledgments

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