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Effect of Cyclothiazide on Binding Properties of AMPA-Type Glutamate Receptors: Lack of Competition between Cyclothiazide and GYKI 52466

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SUMMARY

The effects of cyclothiazide on the properties of (*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors were studied using equilibrium binding techniques and interactions with other compounds known to modulate the receptors. Cyclothiazide caused a reduction in [^{3}H]AMPA binding in assays carried out in the presence of thiocyanate, a chaotropic ion that markedly increases the affinity of AMPA receptors and accelerates their desensitization. In the absence of thiocyanate, however, cyclothiazide had no reliable effect on the binding of [^{3}H]AMPA or on the affinity for this agonist assessed from the displacement of [^{3}H]CNQX. The interaction of cyclothiazide with the receptor appears not to be changed by the presence of thiocyanate. Analysis of the results with a kinetic model of the AMPA receptor suggests that cyclothiazide does not block receptor desensitization by making the desensitized state inaccessible but rather by stabilizing the active state, i.e., by increasing the affinity of the latter to a point where it becomes energetically more favorable than the desensitized state. GYKI 52466, an atypical benzodiazepine that blocks AMPA receptor-gated currents, did not reverse the changes in binding affinity produced by cyclothiazide in the presence of thiocyanate. Physiological experiments conducted in excised patches collected from hippocampal pyramidal cells indicated that thiocyanate does not block access of GYKI 52466 to AMPA receptors. These results point to the conclusion that cyclothiazide acts at a site on the AMPA receptor different from that for GYKI 52466.

The benzothiadiazide compound CTZ causes a profound increase in the duration of AMPA receptor-gated currents (1–4) and a corresponding prolongation of responses at glutamatergic synapses (5). These results, together with those obtained with other drugs recently discovered to modulate AMPA receptors, have provided considerable insight into the relationships between receptor kinetics and the waveform of fast, excitatory synaptic transmission in the central nervous system. They are also of interest because of the possibility that facilitation of AMPA receptor functioning will have therapeutic benefits in a variety of circumstances. In accordance with this, recent studies have shown that centrally active AMPA receptor modulators improve memory encoding in both young and aged rats (6–11). The value of the drugs as experimental tools and as potential therapeutic agents emphasizes the importance of defining the mechanisms by which they enhance AMPA receptor functioning.

Cyclothiazide is very effective in completely blocking the desensitization of responses to AMPA, quisqualate, and glutamate when tested in excised patches and single cells. The desensitized state of a receptor is usually thought to have a higher affinity for agonists than the active state, i.e., the state that is entered initially as the ligand binds and from which the open state is accessed. It is the higher affinity, and thus the lower energy level, of the desensitized state that presumably drives the conversion into the desensitized state. If a drug in some manner prevents the receptor from entering into the desensitized state, then binding tests should reveal the interaction of the ligand with the active states and binding affinity would be expected to be reduced. This was found to be the case in studies in which the effects of CTZ were tested on [^{3}H]AMPA binding, i.e., the drug increased the K_d for AMPA at least 5-fold while producing little change in the B_{max} (12). The EC_{50} for CTZ in the binding experiments was in the range of 30 μM, which is within a factor of 2–3 of that measured in electrophysiological recordings (1). These first studies on receptor affinity were carried out at 0° and in the presence of SCN^- (thiocyanate). The latter is a chaotropic ion that is commonly included in [^{3}H]AMPA binding measurements because it lowers the K_d from 20–40 μM to <1 μM (13). Although this is extremely useful, results from recent studies

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ABBREVIATIONS: AMPA, (*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; GYKI, GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine); SCN^-, thiocyanate; CTZ, cyclothiazide.

strongly suggest that thiocyanate accelerates the conversion of the AMPA receptor to its desensitized state (14, 15). The possibility thus arises that the effects of CTZ may be somewhat different in the presence of thiocyanate than under the conditions used in physiological experiments. The first goal of the present study was thus to assess the influence of CTZ on the binding properties of the AMPA receptor in the absence of SCN^- and at a physiological temperature.

Little is known about the binding sites on the AMPA receptor for CTZ and other modulatory drugs. However, recent work suggests that CTZ reverses the effects of GYKI 52466 (16, 17), an atypical benzodiazepine drug that blocks AMPA receptor-gated currents (16–19) and excitatory transmission in the hippocampus (20). The inhibitory action of GYKI 52466 is noncompetitive with regard to agonists (17), indicating that it is not achieved by blocking access of the transmitter to the receptor. The interaction between CTZ and GYKI 52466 raises the possibility that the two drugs bind to the same site or that the binding site of GYKI 52466 is in close proximity to that of CTZ so that the drugs sterically compete with each other. In either case, one would expect the effects of CTZ on the binding to be reversed in a competitive manner by high concentrations of GYKI 52466. This point was tested in the second part of the present study.

Materials and Methods

Preparation of membranes. Male Sprague-Dawley rats were anesthetized with metofane and killed by decapitation. Membranes were prepared from the telencephalon as previously described (13). In brief, each brain was homogenized in 10 ml of ice-cold 300 mM sucrose and 1 mM EGTA, pH 7.4, in a glass/Teflon homogenizer. The homogenate was centrifuged for 10 min at 1000 × *g*, and the supernatant was then recentrifuged at 30,000 × *g* for 20 min. The resulting pellet was suspended in lysis buffer (1 mM EGTA-Tris, pH 8.0) and left on ice for 30 min. The lysed membranes were collected by a 30-min centrifugation at 40,000 × *g*, and the lysis/centrifugation step was repeated one more time. Membranes were then suspended and washed twice in a Tris-acetate (100 mM, pH 7.4) buffer. After each of these centrifugations, the membrane suspension was sonicated with a tip sonicator at high intensity. The final pellet was suspended in the assay buffer (100 mM HEPES-Tris, 50 μM EGTA, pH 7.4) used for all binding experiments. Membranes prepared from 5–10 rat brains were combined and stored frozen in aliquots at −80°. After an aliquot was thawed, the membranes were sonicated, spun down, and resuspended in assay buffer.

Binding assays. Binding tests were carried out at a temperature of 25°. Aliquots of the membrane suspension (200 μl volume, containing 40–60 μg of protein) were incubated in a microcentrifuge tube with [^{3}H]AMPA for 45 min or with [^{3}H]CNQX for 30 min. Sets of 24 samples were then centrifuged at 25,000 × *g* in a Beckman JA-18 rotor for 20 min; rotor temperature was maintained at 25 ± 3°C during the centrifugation. After the samples were left for 20 min at 25°, the supernatant was aspirated, and the pellet was quickly rinsed superficially with ice-cold buffer containing 50 mM KSCN. Pellets were dissolved in 10 μl tissue solubilizer (Beckman), and their radioactivity content was counted after the addition of acidified scintillation fluid. A quasirandom sequence was adopted for the preparation of the samples, starting of the incubation, and termination of the incubation (aspiration of supernatant and washing of the pellets) so that drug-containing samples were interspersed between control samples. Data were analyzed within a set and expressed relative to the control group within each set. Background values (nonspecific binding) were determined by addition of 5 mM L-glutamate to the incubation and were included in each set. Background values were monitored separately for control and drug-containing samples; these values usually differed by <3%. Cyclothiazide and GYKI 52466 were dissolved in dimethylsulfoxide to prepare stock solutions of 100 and 20 mM, respectively. Protein content was determined according to Bradford (21) with the reagent available from Bio-Rad and with bovine serum albumin as standard; membrane samples were diluted into 0.2 N NaOH before the addition of the dye-reagent.

Patch-clamp recordings in excised patches. Outside-out patches were excised from pyramidal neurons in field CA3 of cultured hippocampal slices and transferred to a separate recording chamber perfused with a recording medium containing 135 mM NaCl, 4.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 0.01 mM MK-801, and 0.02 mM D-AP5. Patch electrodes typically showed a resistance of 5–8 MΩ and were filled with a solution containing 140 mM CsCl, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP disodium salt, and 10 mM HEPES, pH 7.3. The patches were positioned near the septum of a θ-shaped bifurcated glass pipette that provided at any one time either a background flow of recording medium through one of the lines or recording medium plus agonist through the other; the flow was changed from one line to the other by fast-switching valves. The solution exchange time was 0.6 ± 0.1 msec according to junction potential measurements. Holding potentials were in most cases −50 mV. Data were recorded with a patch amplifier (AxoPatch-1D) and digitized at 10 kHz with PClamp (Axon Instruments) on-line.

Materials. [^{3}H]AMPA and [^{3}H]CNQX were obtained from DuPont-NEN. Cyclothiazide was kindly provided by Dr. G. Rogers (Santa Barbara, CA). GYKI 52466 was obtained from Research Biochemicals, CNQX was from Cambridge Research Biochemicals, and AMPA was from Tocris Cookson. Other reagents were from usual commercial sources.

Results

Effects of CTZ. Cyclothiazide has been shown previously to reduce the binding affinity for [^{3}H]AMPA ~5-fold with an EC_{50} of 30 μM (12). A similar although slightly reduced effect was found in the present series of experiments when assays were carried out at 25° instead of 0°. Cyclothiazide inhibited [^{3}H]AMPA binding (at 20 nM radioligand) with an EC_{50} of 46 μM and a maximum extent of inhibition of 72% (not shown). A very different result was obtained when thiocyanate was omitted from the incubations; in this case, [^{3}H]AMPA binding was reduced by <10% at drug concentrations as high as 100 μM (Fig. 1A). Unfortunately, [^{3}H]AMPA binding under these conditions is small and it is not clear to what extent it involves binding to the small subpopulation of high affinity sites typically present in brain membranes (13). For this reason, affinities for AMPA were estimated by measuring the displacement of [^{3}H]CNQX. The latter is an antagonist of both AMPA and kainate receptors but, under the assay conditions used here, ~98% of its binding occurs at AMPA receptors (22). CNQX binding offers two major advantages over AMPA binding: it is much less influenced by SCN^- and does not show a preference for receptors with high affinity for agonists (13, 23). Fig. 1B summarizes the results of a typical experiment in which the effects of AMPA on [^{3}H]CNQX binding were measured. In the absence of drug, AMPA displaced [^{3}H]CNQX with an IC_{50} of 28.8 ± 1.7 μM (K_d values for AMPA derived from the IC_{50} values are given in the legend to Fig. 1; five experiments). This value is similar to that reported earlier for the low affinity site (13); no attempt was made in this case to resolve the small contribution of the high affinity sites, which have an affinity in the range of 1 μM (13). When 200 μM CTZ was included in the assay, the displace-

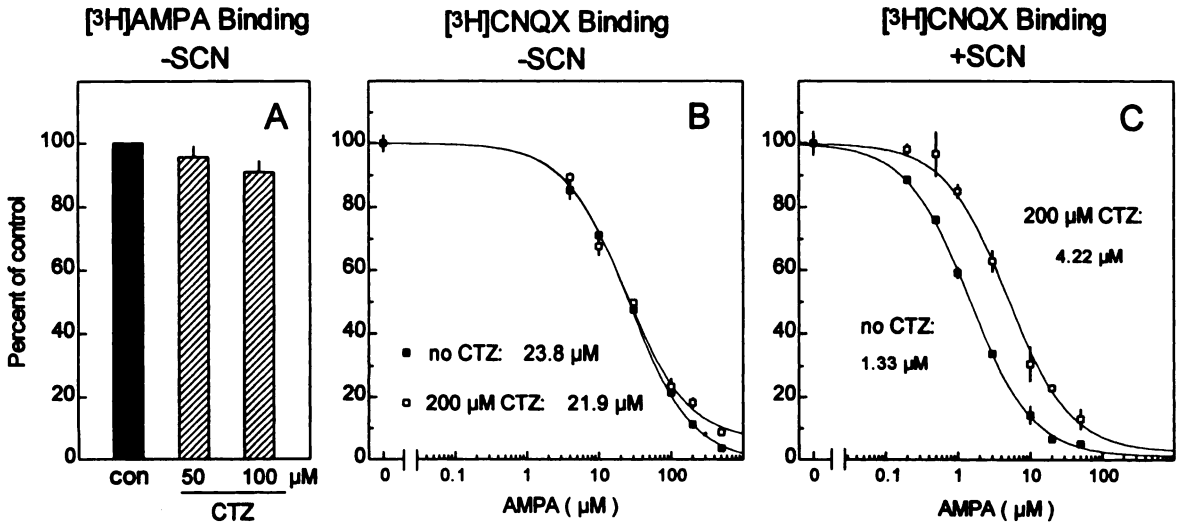

Fig. 1. Effect of CTZ on the binding affinity for AMPA. A, Effect of 50 and 100 μM CTZ on [^{3}H]AMPA (40 nM) binding in the absence of SCN^-. Data are averages from seven or eight paired comparisons with and without drug; error bars indicate the standard error. B, Membranes were incubated in the absence or presence of 200 μM CTZ with 40 nM [^{3}H]CNQX plus one of the AMPA concentrations indicated on the *x*-axis. Measurements at 0 μM AMPA were done in sextuplicate; those at other AMPA concentrations were done in duplicate or triplicate. Data are mean values and standard error from a typical experiment; *inset*, K_d values for AMPA obtained for that particular experiment. Average K_d values from five experiments carried out in tandem were 25.3 ± 1.0 μM in controls and 22.3 ± 3.3 μM in the presence of CTZ; the difference is statistically not significant. K_d values were calculated by multiplying the IC_{50} values obtained from curve fits with Cheng-Prusoff correction factors of 0.88 and 0.94, respectively (see Fig. 3 for K_d values of CNQX binding). Binding at 0 μM AMPA was on average 2.3 pmol/mg protein for controls and 1.08 pmol/mg for CTZ-containing samples. C, Same experiment as in B except that 50 mM KSCN was included in the assay. Average K_d values from three experiments were 1.3 ± 0.1 μM in controls and 4.7 ± 0.8 μM in the presence of CTZ (p = 0.014). Binding at 0 μM AMPA was on average 1.32 pmol/mg for controls and 0.50 pmol/mg for CTZ-containing samples.

ment of [^{3}H]CNQX by AMPA showed essentially the same profile with the possible exception of a small component that appears to become less sensitive to AMPA. The IC_{50} in the presence of drug was 23.7 ± 3.5 μM (five experiments), which is not significantly different from the control value. Similar results were obtained with glutamate instead of AMPA (not shown). Fig. 1C illustrates that measurements of [^{3}H]CNQX displacement provide legitimate estimates of agonist affinity. The experiments were the same as in Fig. 1B except that binding was measured in the presence of SCN^-. In this case, CTZ shifted the IC_{50} of the AMPA displacement curve from 1.4 to 4.8 μM. This ~3-fold shift matches the decrease in [^{3}H]AMPA binding typically seen after the addition of CTZ.

One reason for the lesser effect in the absence of SCN^- could be that the affinity of the receptor for CTZ is greatly reduced in the absence of this ion. This seems unlikely given that physiological experiments on the effects of the drug are conducted without thiocyanate. Additional evidence is given by the experiment represented by Fig. 2 in which the effect of CTZ was measured on [^{3}H]CNQX binding. As is evident, CTZ influenced [^{3}H]CNQX binding in a manner similar to that seen with [^{3}H]AMPA binding, i.e., it caused a reduction of ~50%. More relevant for the present context, however, the EC_{50} for the inhibition was almost the same in the absence (27.4 μM) and in the presence (23.5 μM) of SCN^-. This indicates that the association of CTZ with the receptor is probably not influenced to any large degree by thiocyanate.

Because [^{3}H]CNQX binding is reduced by CTZ, the results shown in Fig. 1, B and C, could be interpreted to mean that the drug binds to only a subset of ~50–70% of the receptors but reduces the affinity of that subset for [^{3}H]CNQX, and presumably AMPA, to an extent that they become virtually undetectable in binding tests. The remaining, CTZ-insensitive population of receptors would then not be expected to reveal any change in their affinity for AMPA or CNQX. A situation of this kind should manifest itself in a large reduction of the B_{max} value in saturation plots. No such change was observed for [^{3}H]CNQX binding. Fig. 3A shows that CTZ reduced the affinity for [^{3}H]CNQX by a factor of ~2 while leaving the B_{max} unchanged; the absence of concave curva-

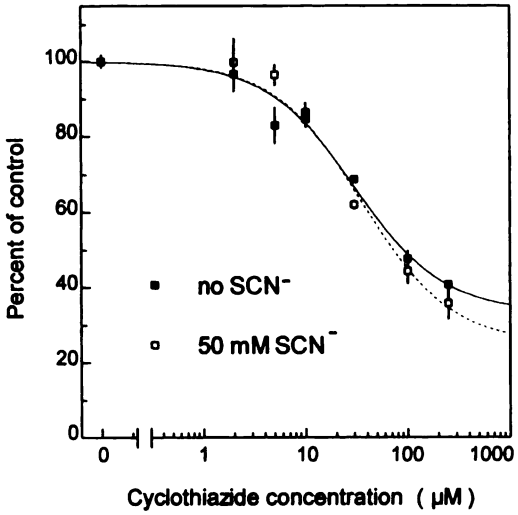

Fig. 2. Effect of CTZ on [^{3}H]CNQX binding in the presence and absence of SCN^-. Membranes were incubated for 30 min with 40 nM [^{3}H]CNQX and either 0 or 50 mM KSCN, plus the CTZ concentrations indicated on the *x*-axis. The figure shows one of four experiments; data points are mean values with their standard error from triplicate determinations. The data points were fitted with sigmoidal inhibition curves with n_H = 1. Average EC_{50} values from four experiments are 27.4 ± 1.5 pmol/mg protein at 0 mM and 23.5 ± 2.0 pmol/mg at 50 mM KSCN; binding was maximally reduced by 63.5 ± 2.5% at 0 mM and by 63 ± 3% at 50 mM KSCN.

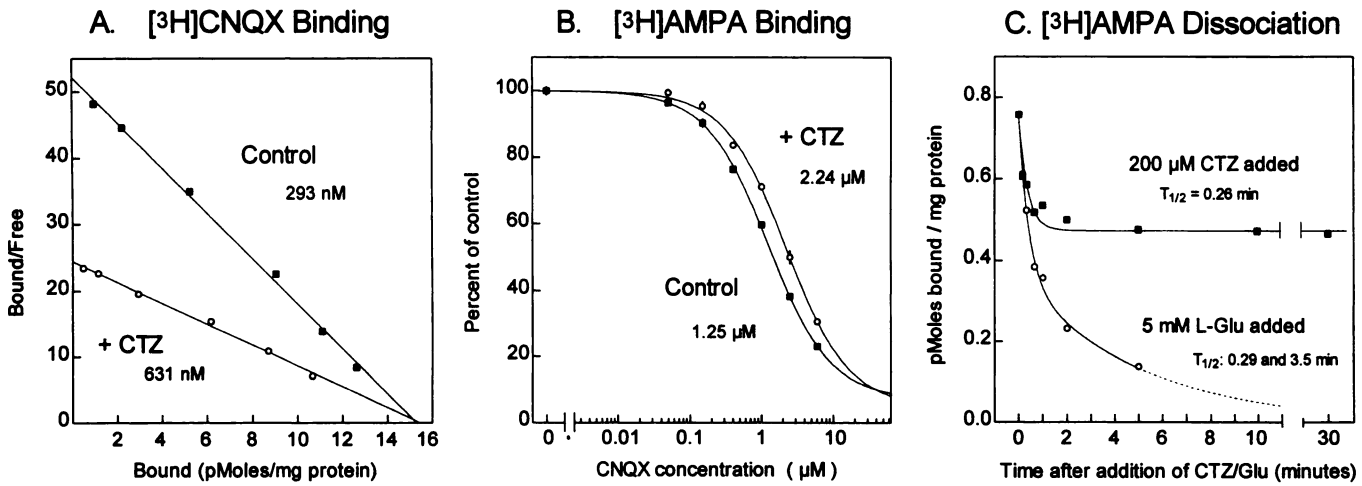

Fig. 3. Effect of CTZ on the binding affinity for CNQX. A, Scatchard plot of [^{3}H]CNQX binding in the absence and presence of 100 μM CTZ. Binding was measured at 20, 50, 150, 400, 800, and 1500 nM CNQX; radiolabeled CNQX was kept at 20 nM, and higher concentrations were achieved by addition of unlabeled CNQX. The figure shows one of three experiments; *insets*, K_d and B_{max} values for that particular experiment. On average, the K_d was increased by CTZ by a factor of 1.9 ± 0.1, and the B_{max} was changed by a factor of 0.94 ± 0.05. K_d values are higher than those reported in other studies because CNQX binding is strongly temperature dependent. B, Displacement of [^{3}H]AMPA binding by CNQX at 0 and 100 μM CTZ. Binding was measured at 20 nM [^{3}H]AMPA in the presence of 50 mM KSCN. *Insets*, EC_{50} values; Cheng-Prusoff corrections were not applied because they would produce only minor adjustments. C, Time course for the adjustment of [^{3}H]AMPA binding after the addition of 200 μM CTZ. Membranes were allowed to equilibrate for 45 min at 25° with 20 nM [^{3}H]AMPA in the presence of 50 mM KSCN. At time t = 0, CTZ or 5 mM glutamate was added to the incubation. Incubations were terminated at the indicated time points by dilution in ice-cold stop buffer (assay buffer plus 50 mM KSCN) and filtration through GF/C glass-fiber filters; the filters were washed three times with stop buffer. The data points were fitted with single and double exponential decay functions; *insets*, the time constants of the relaxation curves. Because dissociation forced by CTZ and that forced by glutamate had similar time constants, the effect of CTZ may have been limited by the dissociation of bound [^{3}H]AMPA. Dissociation forced by glutamate is biphasic due to the presence of high and low affinity binding sites in the membrane preparation (13, 15).

ture suggests that the drug acts in a similar manner on most AMPA receptors present in the membrane preparation. Similar conclusions were made in the earlier study of Hall *et al.* (12) with regard to [^{3}H]AMPA binding. It is further supported by Fig. 3B, which shows that the binding of [^{3}H]AMPA that remained in the presence of 100 μM CTZ had a 2-fold lower affinity for CNQX. This indicates that the same receptors that were carrying the [^{3}H]AMPA had bound a CTZ molecule since their affinity for CNQX was reduced by approximately the amount predicted based on Fig. 2.

In a recent study in which binding of a radiolabeled GYKI analog to *Xenopus* brain membranes was characterized, Szabo and Henley (24) reported that optimal binding requires incubation times of several hours at ambient temperature. If this were true for CTZ as well, and patch-clamp studies have shown that its binding and unbinding are slow compared with those of other AMPA receptor ligands, then the effects of the drug could be underestimated with our binding protocol. Fig. 3C suggests that this is not the case. When CTZ was added to a binding incubation at its equilibrium, [^{3}H]AMPA binding approached a stable new equilibrium within ~2 min.

Effects of GYKI 52466. Table 1 shows that even high concentrations (200–400 μM) of GYKI have no detectable influence on [^{3}H]AMPA binding. A small but reliable increase was observed, however, in the binding of [^{3}H]CNQX. The dose-response measurements shown in Fig. 4 indicate that the increase reaches a maximum of ~8% at GYKI concentrations of >50 μM; the EC_{50} is 16 μM, which is similar in magnitude to values reported from physiological studies (17). Because the effect on [^{3}H]CNQX binding is small, it is difficult to obtain reliable information concerning target and mode of action of GYKI. Glycine at 10 μM inhibited

TABLE 1
Effect of GYKI 52466 on [^{3}H]AMPA and [^{3}H]CNQX binding

Ligand	±SCN	Concentration	Change from control	*n*
		μM	%	
[^{3}H]AMPA binding	−	200–400	−1.6 ± 3.7	9
	+	200–400	3.1 ± 3.8	6
[^{3}H]CNQX binding	−	200	7.3 ± 0.8[b]	11
	+	200	6.2 ± 0.7[a]	3

Effect of GYKI 52466 on the binding of 20 nM [^{3}H]AMPA and 40 nM [^{3}H]CNQX in the presence and absence of 50 mM KSCN. Values are mean ± standard error from *n* determinations, with each determination carried out in quadruplicate to sextuplicate. Binding assays were carried out at 25°. For [^{3}H]AMPA binding, data collected at 200 and 400 μM drug concentrations were combined.
[a] $p < 0.05$, [b] $p < 0.001$.

[^{3}H]CNQX binding by <1% in the absence or presence of GYKI (not shown); therefore, the observed effects are not likely to result from an action on NMDA receptors. Binding to kainate receptors has been estimated to account for ~2% of [^{3}H]CNQX binding under the assay conditions used here (22); relative contributions from kainate and AMPA receptors can not be easily assessed, however, because a major subpopulation of the latter also exhibits relatively high affinity for kainate (22). Notwithstanding these limitations, the results indicate that GYKI binds to receptors in our membrane preparation and does so with the expected affinity.

If GYKI and CTZ act through the same site then the effects of the latter should be reversed by high concentrations of the former and the dose-effect curves for CTZ should be shifted toward higher concentrations. Two experiments of this kind are illustrated in Fig. 5, in which [^{3}H]AMPA and [^{3}H]CNQX binding were measured with increasing CTZ concentrations in the absence and presence of 200 μM GYKI; assays with [^{3}H]AMPA were done in the presence of SCN^-, and those with [^{3}H]CNQX were done in its absence. It is evident from

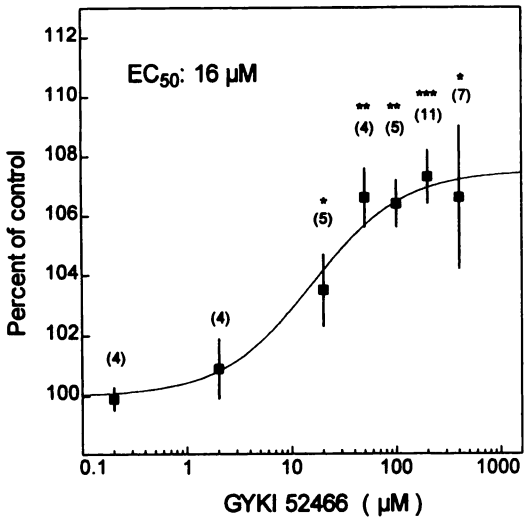

Fig. 4. Effect of GYKI 52466 on [^{3}H]CNQX binding. [^{3}H]CNQX binding was measured at a concentration of 40 nM in the absence and in the presence of the GYKI concentrations indicated on the *x*-axis; no SCN^- was present. Nonspecific binding was determined by inclusion of 5 mM L-glutamate. The data show the percent increase over controls. *Numbers in parentheses*, number of pairwise comparisons between control and drug samples; in each such comparison, measurements were done in quadruplicate to sextuplicate with separate background values with and without drug. GYKI was added from 100-fold (final, 0.2–200 μM) or 50-fold (400 μM) concentrated solutions in DMSO; control samples received the equivalent concentration of DMSO. The data points were fitted with a logistic function (n_H = 1); the EC_{50} obtained from this curve fit is 16.0 μM. Data points indicated by asterisks are significantly different from control (*, $p < .05$; **, $p < .01$; ***, $p < .001$).

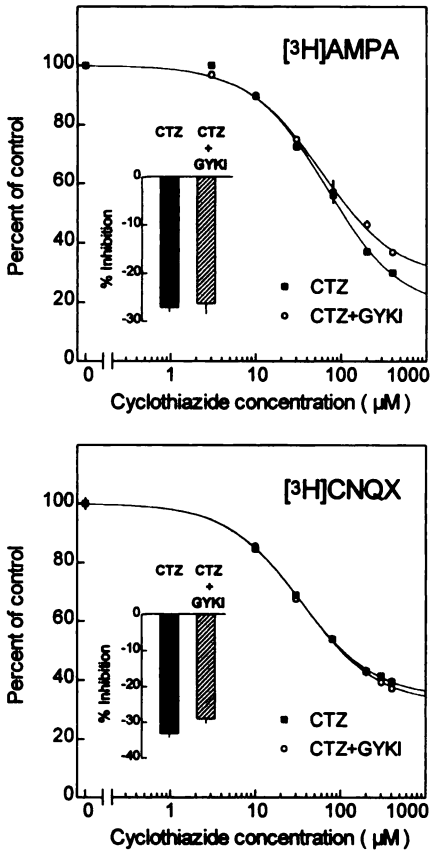

Fig. 5. Effects of CTZ on [^{3}H]AMPA and [^{3}H]CNQX binding in the presence and absence of GYKI. *Top*, Binding of 20 nM [^{3}H]AMPA was measured in the presence of 50 mM KSCN, the CTZ concentrations shown on the *x*-axis, and either 0 or 200 μM GYKI. Measurements were in triplicate. The EC_{50} values for CTZ were 57 μM in the absence and 66 μM in the presence of GYKI. Essentially identical results were obtained in a second experiment (see text). Averaged data from seven experiments in which inhibition was measured at fixed CTZ concentration of 30 μM and at 200 or 400 μM GYKI are shown (*inset*, bar graphs). *Bottom*, same as in *top* except that the effects on 40 nM [^{3}H]CNQX binding were measured in the absence of SCN^-. EC_{50} values for the curves shown in this graph are 33 μM without GYKI and 35 μM in presence of 200 μM GYKI. *Inset*, averaged data from five measurements at 30 μM CTZ and 200 or 400 μM GYKI.

both sets of experiments that GYKI does not produce any noticeable shift in the CTZ inhibition curve. In the case of [^{3}H]AMPA binding, EC_{50} values for CTZ in two experiments were 57 and 46 μM without and 66 and 56 μM in the presence of GYKI. For [^{3}H]CNQX binding, the respective EC_{50} values were 37 and 33 μM without GYKI and 37 and 35 μM in the presence of GYKI. Data from a larger set of experiments in which the inhibition by a single intermediate concentration of CTZ (30 μM) was measured without and with GYKI (Fig. 5, *insets*) lead to the same conclusion. Taken together, the data strongly suggest that GYKI does not reverse the effects produced by CTZ in binding experiments.

A complicating aspect in some experiments might have been the possibility that SCN^- allosterically reduces the affinity for GYKI or that it displaced GYKI from its binding site. Evidence against such an interaction is given by the experiment represented by Fig. 6, which shows that GYKI inhibited AMPA-induced currents to a similar extent in the presence and absence of SCN^-. Fast application of AMPA to patches excised from hippocampal pyramidal cells produced responses that reached a peak within a few milliseconds and then declined to a much lower steady state with a time constant of ~10 msec (15). The bottom row of the figure shows responses in the same patch in the presence of SCN^-; in accordance with our previous report (15), amplitude and decay time constant of the control responses were reduced by SCN^- by 47% and 55%, respectively. In the absence of chaotropic ions, 50 μM GYKI reduced the peak amplitude of the response to 17 ± 11% of the control (± standard deviation; two experiments). In the presence of SCN^-, the peak amplitude was reduced to 16 ± 1% of the corresponding control response (i.e., that measured in the presence of SCN^- alone). This indicates that the inhibition by GYKI is not significantly altered by the presence of SCN^-.

Discussion

Vyklicky *et al.* (25) introduced a kinetic model of the AMPA receptor that adequately reproduces the time course of AMPA receptor-mediated responses and, in particular, the fast desensitization that leads to a small steady state current (~10% of the peak current). This model assumes that the receptor exists in five different kinetic states as shown in Fig. 7A, i.e., the three states [R (unbound receptor), RA (receptor/ligand complex), and O (open channel state)] that can be called the active states and the two desensitized states (R* and R*A). An isomorphic model with a slightly altered set of rate constants was shown by Ambros-Ingerson and Lynch (26) to reproduce a variety of other observational parameters, including mean open and closed times, binding constants, drug effects, and the waveform of synaptic currents. Any

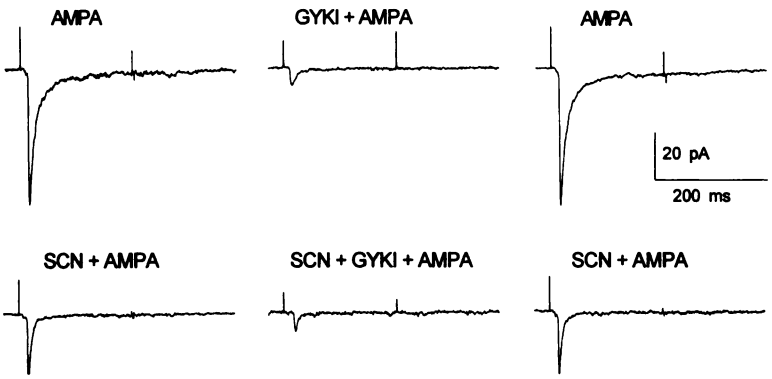

Fig. 6. Effect of GYKI on AMPA-induced responses in excised patches in the presence and absence of SCN^-. Representative experiment illustrating the effects of GYKI 52466. Inward currents were induced by a 200-msec pulse of 2 mM AMPA, which produces a near-maximal peak current. GYKI 52466 and thiocyanate were included in both background and agonist-containing flow lines. Each trace corresponds to the average of five consecutive responses; the records shown in this figure were collected from the same patch at intervals of 30 sec. The holding potential was −50 mV. *Top*, traces taken before, during, and after application of 50 μM GYKI. *Bottom*, effect of 50 μM GYKI in the presence of 10 mM thiocyanate. Sodium thiocyanate reversibly reduced the peak current and the desensitization time constant by 47% and 55%, respectively. GYKI (50 μM) decreased the peak current to a similar extent as in the absence of thiocyanate.

such model that attempts to reproduce the commonly observed large desensitization must incorporate a difference in magnitude between the desensitization rate k_2 and the resensitization rate k_{-2}, as well as an asymmetry of comparable magnitude in the binding affinities of the desensitized versus the active states. The dissociation constant K_d, which governs the binding properties of the receptor, is a function of all of the independent rate constants; in the five-state model, it is given by the equation $K_d = (R_1 + R_3/R_2)/(1 + 1/R_2 + 1/R_5)$, where each R_i is the ratio of the rate constants k_{-i}/k_i (26). For the set of rate constants adopted in Fig. 7, the K_d value is 28 μM. Without the states R* and R*A, the formula for the dissociation constant is reduced to $K_d = R_1/(1 + 1/R_5)$. Thus, if a drug would prevent access to the desensitized states but leave events between the three active states R, RA, and O kinetically unchanged, the K_d would be ~800 μM, i.e., >10 times larger than when desensitization can occur. A difference of this magnitude can be discerned in the EC_{50} values for early and late events after step application of glutamate. Peak currents, which are largely determined by the transitions among the active states, saturate with EC_{50} values in the range of 300-1000 μM (15, 27), whereas the steady state currents, which like binding measures are determined by equilibrium distribution among all five receptor states, saturate with an EC_{50} in the order of 5–10 μM (27, 28).

Considerations of this nature have been previously used to explain the observation (12) that [^{3}H]AMPA binding was greatly reduced in the presence of CTZ. As described, the present study was undertaken to examine whether a similar shift in affinity occurs when the assays are carried out in the absence of SCN^- and at 25°. The results indicate that this is not the case. Thus, the idea that CTZ simply renders the desensitized state inaccessible is not supported by the new evidence. If CTZ prevents desensitization yet does not change binding affinity, then an intuitively plausible explanation would be that the drug increases the affinity of the active (i.e., nondesensitized) states such that they become more similar to the desensitized state. Considerations of this kind are summarized in Fig. 7, which compares these two ways in which a modulator might prevent the receptor from entering the desensitized state. Calculations have been carried out with the model of Fig. 7A and with the assumption that a drug changes the indicated rate constants by a factor of 100.

Case I corresponds to the situation in which the drug renders the desensitized state unfavorable. This implies that the free energy level of R*A is increased to an extent that the presence of the receptor in this state becomes a less likely event. This would occur if the ratio k_{-2}/k_2 increases (i.e., if the desensitization rate is reduced, the resensitization rate is increased, or any combination) and if it is balanced by a concomitant change in k_{-3}/k_3 (or in k_{-4}/k_4) to satisfy microreversibility. With a 100-fold change in these constants, binding affinity decreases 27 times (row C). As the rate constants connecting R, RA, and O are assumed not to be changed, the dose-response profile for the peak current experiences minimal changes. Case II shows the alternative situation in which the energy of the active state, and with it that of the open state, is reduced so they become as favorable as the desensitized state. In this case, a 100-fold increase in the ratio k_{-2}/k_2 would have to be balanced with an equal size decrease in the ratio k_{-1}/k_1; i.e., the dissociation constant governing the binding of the agonist to the active state would be decreased. In this situation, equilibrium binding affinity is increased rather than reduced, but it should be noted that the magnitude of the change is much smaller than in case I. In clear distinction from case I, the dose-response curve for the peak current shifts to the left by ~2 orders of magnitude. An additional criterion with which to distinguish between the two cases could be provided by measurements of the affinity for CTZ as each state of the receptor may have a different affinity for the drug. As indicated (Fig. 7, *Row E*), the addition of glutamate should reduce the affinity for CTZ in case I but increase it in case II.

Changes of both types, if sufficiently large (>50 fold)), could lead to nondesensitizing responses of the kind produced by CTZ. Our observation that binding affinity is not changed or, if anything, slightly increased strongly favors model II. This conclusion is further supported by the dose-response relationships of the peak current (Fig. 7, *Row D*), Yamada and Tang (1) reported that the EC_{50} for currents produced by quisqualate changed from 280 μM to ~1 μM on the addition of 10 μM CTZ. A similar shift was observed in our experiments using glutamate as an agonist (not shown). The prediction with regard to drug binding could not be tested because CTZ is not available in radiolabeled form.

Effects similar to those of case II would also be produced if the drug would preferentially stabilize the open state but not the state RL, i.e., if it would accelerate the opening rate k_5 and/or slow down the closing rate k_{-5}. A change in these constants alone is unlikely, however, because the ratio between k_5 and k_{-5} would have to increase ~500-fold to eliminate desensitization. A change of this magnitude should produce a dramatic increase in the off-rate, i.e., in the rate at which the response decays at the offset of the glutamate pulse, whereas the experimentally determined values show minimal changes (1, 3).

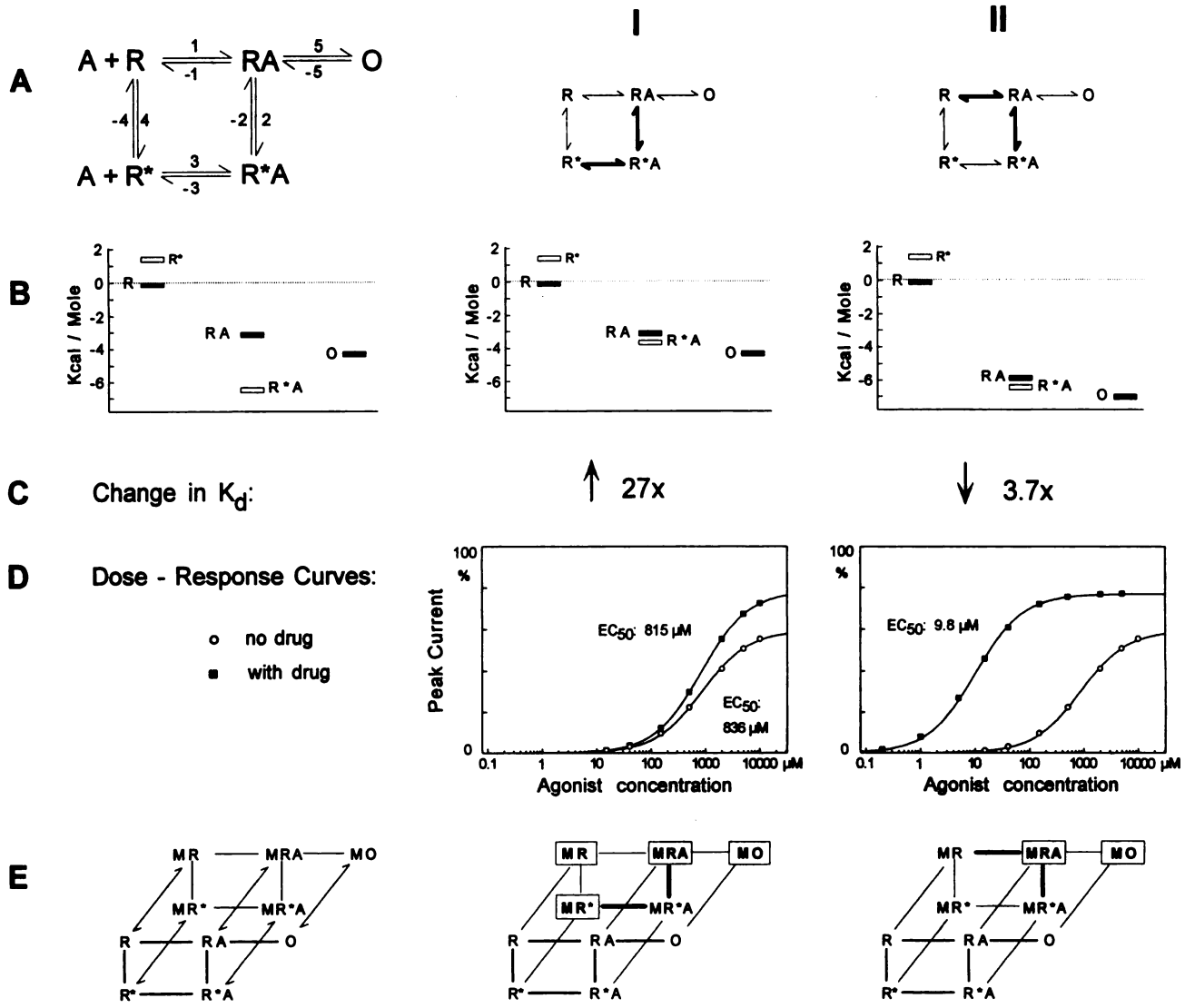

Fig. 7. Interpretation of binding data within a five-state AMPA receptor model. A, Five-state receptor model as described elsewhere (15, 26). *R*, the receptor; *A*, an agonist such as L-glutamate or AMPA; *O*, the open state. *, Desensitized states. *Numbers next to transition arrows*, number of the rate constants; the rate constants were selected as k_1 = 1.5 $\mu M^{-1}\cdot sec^{-1}$; k_3 = 5 $\mu M^{-1}\cdot sec^{-1}$; and k_{-1} = 10,000 sec^{-1}, k_2 = 1500 sec^{-1}, k_{-2} = 6 sec^{-1}, k_{-3} = 10 sec^{-1}, k_4 = 3 sec^{-1}, k_{-4} = 40 sec^{-1}, k_5 = 5000 sec^{-1}, and k_{-5} = 700 sec^{-1}. *Schemes in columns I and II*, sets of rate constants that are changed in these two special cases; in case I, for example, the ratio k_{-2}/k_2 would be changed by a factor of 100 by changing either or both of these rate constants. *Left column*, condition in the absence of drug. B, Schematic of the free energy levels of the different receptor states calculated from the equilibrium constants connected to each state. The energies of the different states were calculated relative to that of the R state. *Vertical axis*, energy levels; *horizontal displacements*, used only for visualizing the different receptor states; *bold*, active states; *empty rectangles*, desensitized states. The *y*-axis is calibrated in kcal/mol. Energy levels were calculated according to the equation $\Delta G = RT \ln K_d$ with RT = 0.592 kcal/mol (at 25°), and rate constants are expressed in sec^{-1} and mol^{-1}. C, Change in the dissociation constant of equilibrium ligand binding for 100-fold changes in the rate constants as indicated in A. *Upward arrow*, increase in the K_d, i.e., a decrease in binding affinity. D, Dose-response curves for the peak current in the absence of drug (circles) and for cases I and II where the drug is assumed to produce 100-fold changes in a subset of the rate constants. Currents induced by 200-msec application of the agonist concentrations indicated on the *x*-axis were simulated with the 2 × 5-state model shown in E; peak currents were then plotted against agonist concentration. For these calculations, the desensitization rate constant was reduced by a factor of 10, and the resensitization constant was multiplied by 10; the 100-fold change in the other set of rate constants was similarly divided between forward and backward rate constant. The association rate of the modulator drug was assumed to be 1 $sec^{-1}\cdot\mu M^{-1}$, and the dissociation rate was 1 or 100 sec^{-1} (see E). Units on the *y*-axis represent the percentage of the maximal current that would be obtained if all receptors are conducting; *insets*, EC_{50} values. E, A 2 × 5-state receptor model derived from that in A which features the binding of the modulator drug M to each of the five basic states. This scheme is governed by 30 rate constants with seven loops. *Schemes shown under I and II*, the drug-bound states marked by a *box* must have a 100-fold higher affinity for the drug than the unmarked states to satisfy microreversibility.

The chaotropic ion SCN^- accelerates desensitization of AMPA receptor currents in excised patches and shortens the decay time of hippocampal EPSPs (Fig. 6) (15). These effects can be explained if one assumes that the ion stabilizes the desensitized state R*A by accelerating the transition to the latter and by slowing dissociation of the ligand from the desensitized state by a factor of ~20 (15). An obvious question then is whether CTZ would cause the reduction in binding affinity described in Fig. 1C if the kinetic changes depicted above for case II were applied to a receptor operating under the influence of SCN^-. That this is not the case can be deduced from Fig. 7B if one takes into consideration that

binding affinity is determined to a large extent by the state(s) with the lowest energy level: if SCN were to lower R*A, then the latter would remain the lowest state with or without CTZ and thus binding would be minimally influenced by the latter compound. To mimic the effects of Fig. 1C, one has to assume that CTZ not only lowers RA but also raises the level of R*A such that the latter comes to lie above RA in the combined presence of CTZ and SCN^-. Fig. 8 shows a possible set of SCN^-- and CTZ-induced changes that can adequately reproduce the observed binding effects. It is apparent from this that the change in the ratio k_2/k_{-2} produced by CTZ would have to be much larger than the factor of 100 used for the simulations in Fig. 7.

The two cases outlined in Fig. 7 obviously should be considered as prototype models; actual drug effects may prove to be composites of the hypothesized effects and include changes in other rate constants (see Fig. 8). Nevertheless, the above considerations suggest that a stabilization of the active states RA and O are likely to be among the predominant effects of CTZ. In this regard, the effects of the benzothiadiazide are probably similar to those of other AMPA receptor-enhancing drugs, such as 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine (29) and its analogues. However, it has been observed that CTZ- and benzoylpiperidine-type drugs consistently produce somewhat different effects on receptor binding; e.g., several of the latter compounds cause a marked increase in [^{3}H]AMPA binding in the absence of SCN^-.[1] It thus is not unlikely that a more refined kinetic analysis will reveal distinctive aspects in the ways different families of compounds influence AMPA receptor functioning.

As demonstrated above, drugs that affect receptor kinetics are likely to modulate binding properties at least under some circumstances and the lack of effect of GYKI in this regard is thus somewhat unusual. Perhaps the only modification in the above receptor model that could eliminate receptor currents without influencing binding affinity (< 3% change) would be a reduction in the opening rate k_5 or an acceleration in the closing rate k_{-5} (or a combination thereof). An obvious alternative is that GYKI blocks ion passage in a manner that does not involve changes in the gating mechanism. What is difficult to explain in either case is why CTZ and GYKI appear to interact in a competitive manner in physiological measurements but do not reveal evidence of such an interaction in binding tests. The GYKI concentration employed here was ten to twenty times above the EC_{50} value reported from physiological experiments and should have been more than sufficient to produce a noticeable shift in the CTZ inhibition curve. GYKI appeared to bind to at least some receptors in the expected concentration range of 10–20 μM which makes it unlikely that receptors in membrane preparations lose their affinity for GYKI. The binding results thus suggest that effects of GYKI and CTZ on AMPA receptors are not mediated by the same site and that the reversal of GYKI inhibition by CTZ is a consequence of opposing influences on receptor kinetics rather than competition. A situation of this kind could arise, for example, if a reduction in the channel opening probability by GYKI would be counteracted by a reduction in desensitization by CTZ. Other evidence against a competitive interaction between the two compounds has recently been reported by Desai *et al.* (30).

Binding measurements and physiological recordings reveal somewhat complementary aspects of receptor kinetics in that the former are mostly concerned with nonstationary and the latter with stationary aspects of receptor function. The data obtained from these two approaches should, however, match if appropriate comparisons are made and if binding data are

[1] M. Kessler, unpublished observations.
[2] A. Arai, unpublished observations.

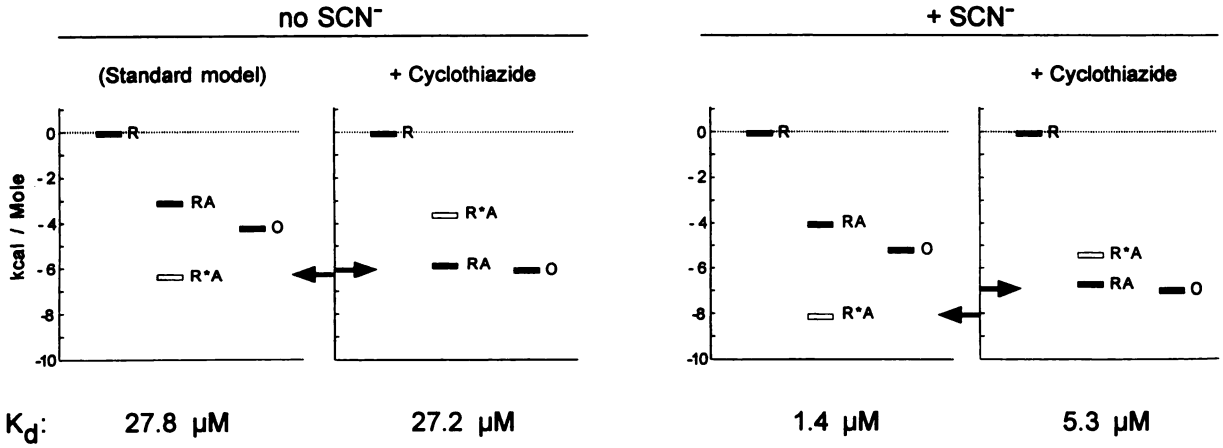

Fig. 8. Schematic of possible receptor changes produced by CTZ in the absence and presence of SCN^-. Shown are plots for the free energy levels of the different receptor states as in Fig. 7B and the K_d values associated with these sets (the graphs do not show R* as its position only affects the balance between $k_{\pm 3}$ and $k_{\pm 4}$). *Left*, standard set of rate constants as introduced in Fig. 7A. *Second from left*, cyclothiazide is assumed to change rate constants by the following factors: $k_1 \times 4$, $k_{-1} \times 0.04$, $k_2 \times 0.001$, $k_{-2} \times 10$, $k_4 \times 0.01$, and $k_5 \times 0.2$; the ratio k_2/k_{-2} changes from 250 to 0.025. R*A therefore comes to lie above RA. *Second from right*, with SCN^-, rate constants are assumed to change as follows: $k_{-1} \times 0.2$, $k_2 \times 4$, and $k_{-3} \times 0.05$. This set of changes produces a reduction in the peak amplitude and the decay time constant by approximately half (compare with Fig. 6); it lowers state R*A and, to a smaller extent, RA and O. The lowering of R*A is the main reason for the affinity to increase to 1.4 μM. *Right*, effects of CTZ in the presence of SCN^-, calculated under the assumption that the effects are independent, i.e., the rate constants were multiplied by the product of the changes listed above for SCN^- and CTZ (*vertical shifts on the ΔG scale* accordingly are additive). CTZ lowers RA but not sufficiently to bring it down to the level of R*A; R*A has been raised. As a consequence, binding affinity is reduced. *Arrows*, position of the lowest energy state. K_d values were calculated with the equation given in the text. The set of rate constants used above for CTZ satisfies the following constraints: (i) responses are nondesensitizing, (ii) the off-rate is changed by a factor of <4 (3),[2] (iii) binding affinity remains unchanged in the absence of SCN^-, and (iv) binding affinity is reduced 3–4-fold when SCN^- is present.

collected under conditions that approximate those used in the physiological experiments. Such agreements have now been demonstrated in several instances. As indicated earlier, K_d values for AMPA and glutamate (20–40 μM) correspond reasonably well with EC_{50} values of steady-state currents. Similarly, the effects of SCN^- on binding can be quantitatively correlated with its effects on AMPA responses in excised patches (15). One conclusion to be drawn from this is that the process of preparing membranes with its disruption of the cellular environment does not produce major disturbances in the way AMPA receptors interact with agonist and antagonists. The data presented above extend this list as they show that the effects of CTZ on binding can be accommodated within existing AMPA receptor models.

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