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ALLOSTERIC REGULATION OF α-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLE-PROPIONATE RECEPTORS BY THIOCYANATE AND CYCLOTHIAZIDE AT A COMMON MODULATORY SITE DISTINCT FROM THAT OF 2,3-BENZODIAZEPINES

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Abstract—Allosteric regulators of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors include 2,3-benzodiazepines such as GYKI 52466 and GYKI 53655 and the chaotropic anion thiocyanate that inhibit, and benzothiadiazines such as cyclothiazide that potentiate AMPA receptor currents. Here we sought to determine whether the allosteric regulators modulate AMPA receptors at a common or distinct allosteric sites by comparing their actions on AMPA- and kainate-evoked currents in cultured rat hippocampal neurons and *Xenopus* oocytes expressing recombinant AMPA receptor subunits. GYKI 52466 and thiocyanate blocked AMPA-evoked currents in a concentration-dependent manner (IC_{50} values, 8.2 μM and 1.1 mM, respectively); in contrast, kainate-evoked currents were blocked by GYKI 52466, but were potentiated by high concentrations of thiocyanate ($\geq$3 mM). Thiocyanate enhanced the rate of desensitization and slowed recovery from desensitization of AMPA-evoked currents, whereas GYKI 52466 failed to affect desensitization. Among neurons in the hippocampal cultures, there was cell-to-cell variability in the sensitivity to block of AMPA-evoked currents by thiocyanate that was correlated with the degree of potentiation by cyclothiazide. Moreover, cyclothiazide caused a parallel rightward shift in the concentration–response curve for thiocyanate block, and slowed the onset of thiocyanate block to a rate that was similar to that of cyclothiazide dissociation. Together, these observations suggest that thiocyanate and cyclothiazide act at non-distinct allosteric sites. GYKI 52466 blocked AMPA receptor responses to a similar extent, irrespective of the degree of cyclothiazide potentiation. Moreover, the kinetics of GYKI 53655 block in the presence of cyclothiazide were not consistent with a competitive interaction. As is the case for cyclothiazide, SCN^- exhibited greater affinity for *flip* than for *flop* AMPA receptor splice variants. In particular, $GluR1_{flip}/GluR2_{flip}$ was especially sensitive to thiocyanate block.

We conclude that thiocyanate, a *flip*-preferring allosteric modulator like cyclothiazide, appears to act by enhancing desensitization at a site that may overlap the site where cyclothiazide reduces desensitization, whereas 2,3-benzodiazepines act at a distinct site and the block does not involve a modification of desensitization.

Key words: AMPA receptor, *flip* and *flop*, 2,3-benzodiazepine, thiocyanate, cyclothiazide, desensitization.

Glutamate acting at α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)-selective glutamate receptors mediates the bulk of fast excitatory neurotransmission in the CNS. In recent years, a variety of pharmacological agents have been identified that allosterically regulate AMPA receptors. These allosteric regulators represent important tools for probing AMPA receptor function, and in addition may be potentially useful as therapeutic agents. The first allosteric modulators to be described were the 2,3-benzodiazepines including GYKI 52466 and its more potent analogue GYKI 53655 which selectively inhibit AMPA receptors by interacting with a site distinct from the glutamate recognition site.[5,7,30] The exact mechanism by which 2,3-benzodiazepines exert their blocking effects is not well understood. Initial studies suggested that 2,3-benzodiazepines might act to enhance AMPA receptor desensitization.[30] This hypothesis was supported by the observation that the benzothiadiazine cyclothiazide, which retards desensitization and potentiates AMPA receptor currents,[20,24,28,30] could reverse the blocking effects of the 2,3-benzodiazepines.[16,24,30] However, electrophysiological studies using rapid perfusion techniques showed that 2,3-benzodiazepines had no effect on the rate of AMPA receptor desensitization[5,25] and it was observed that cyclothiazide and the

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; NMDA, *N*-methyl-D-aspartate.

2,3-benzodiazepines are able to independently access their sites of action.[10,29] The conclusion suggested by these latter studies was that cyclothiazide and the 2,3-benzodiazepines act at distinct sites and the block produced by the 2,3-benzodiazepines is not the result of an enhancement of desensitization.

The chaotropic anion thiocyanate (SCN^-), which enhances [^{3}H]AMPA binding to brain membranes,[2,13–15] has also been shown to act as an allosteric inhibitor of AMPA receptors.[3] As is the case for the 2,3-benzodiazepines, the exact mechanism of block is obscure, although recently SCN^- has been shown to enhance AMPA receptor desensitization.[1,6,19]

In the present study, we compared the actions of SCN^- and the 2,3-benzodiazepines on AMPA- and kainate-evoked currents in cultured rat hippocampal neurons and in *Xenopus* oocytes expressing recombinant AMPA receptor subunits. Our objective was to investigate whether these negative modulators block AMPA receptors at the same or distinct allosteric sites. Moreover, we sought to determine whether either antagonist might interact at a site in common with the positive modulator cyclothiazide.

EXPERIMENTAL PROCEDURES

Hippocampal neuron recording

Cell culture. Hippocampal neurons from 19-day-old Sprague–Dawley rat embryos (Harlan-Sprague–Dawley, Indianapolis, IN) were grown in primary culture as described previously[26] and were used five to eight days after plating.

Whole-cell and excised patch recording. Recordings were carried out at room temperature (23°C) in a bathing solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 mM $MgCl_2$ and 10 HEPES. The bathing solution also contained 1 μM tetrodotoxin to block voltage-activated Na^+ channels. In experiments examining the response to *N*-methyl-D-aspartate (NMDA), bathing solutions were prepared without $MgCl_2$, and 10 μM glycine and 1 μM strychnine were added. Whole-cell and outside-out excised patch (voltage-clamp) recordings were obtained with an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA) using patch electrodes (2–4 MΩ) filled with an intracellular solution containing (in mM) 145 $CsCl_2$, 2 $MgCl_2$, 5 HEPES, 0.1 $CaCl_2$, and 1 EGTA. The holding potential was maintained at −60 mV, unless otherwise noted. Currents were acquired on a graphic recorder and on a microcomputer using the pClamp (Clampex) or Axotape software packages (Axon Instruments).

Drug perfusion. Drug solutions were applied using a rapid perfusion system described previously[5] in which the solution exchange time constant at the bare tip of a recording electrode was less than 2 ms. One barrel contained bathing solution while the other barrels contained agonist and drugs either alone or in combination. SCN^- was applied as the Na^+ salt.

Oocyte recording

Oocyte injection. Female *Xenopus laevis* frogs were anaesthetized by immersion in 0.2% tricaine for 15 to 30 min. The ovarian lobes were removed and incubated with gentle shaking for 2 h at room temperature in solution containing (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES and 2 mg/ml type IA (Sigma) collagenase (pH 7.6). Oocytes were defolliculated and rinsed five to six times in a modified Barth's solution containing (in mM) 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 1 $MgSO_4$, 2.4 $NaHCO_3$ and 10 HEPES (pH 7.4). Selected stage V-VI oocytes were stored for 24 h at 18°C in Barth's solution supplemented with 1 mM Na-pyruvate, 0.01 mg/ml gentamycin, 100 units/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml of amphotericin B. Glass capillary tubes (1 mm o.d.; World Precision Instruments, Sarasota, FL) were drawn to a fine tip with a vertical micropipette puller and broken back to an outside diameter of 20 μm. cRNA was prepared by *in vitro* transcription of linearized cDNA encoding $GluR1_{flip}$, $GluR1_{flop}$, $GluR2_{flip}$, $GluR3_{flip}$ (kindly provided by Dr Steven Heinemann, Salk Institute for Biological Studies, La Jolla, CA). cRNA stocks were diluted to a final concentration of 1 to 2 μg/μl, and 23 to 50 nl of the resulting solution was injected, either alone or in combination, into the oocyte cytoplasm.

Electrophysiology. Two-electrode voltage clamp recordings were performed three to 10 days following injection at room temperature in control Ringer solutions containing (in mM) 115 NaCl, 2.5 KCl, 1.0 $BaCl_2$, and 10 HEPES (pH, 7.4) using 3 mM KCl-filled microelectrodes (1–5 MΩ) and a Geneclamp amplifier (Axon Instruments, Burlingame, CA). The holding potential was −60 mV.

Data analysis

The fractional block (B) was calculated according to the formula $B = 1 - I_B/I$, where I is the steady-state current evoked by agonist and I_B is the current evoked by the agonist in the presence of a blocker. Concentration–effect data were fit to the logistic equation $B = 1/[1+(IC_{50}/[D])]^{nH}$ where $[D]$ is the drug concentration, IC_{50} is the antagonist concentration resulting in 50% block and n_H is an empirical parameter describing the steepness of fit and having the same meaning as the Hill coefficient. EC_{50} values were determined from fits to a logistic equation of a similar form. NFIT (Island Products, Galveston, TX) was used for nonlinear curve fitting. Data are presented as the mean ± S.E.M.; n is the number of cells tested. The statistical significance of differences between population means was assessed with the unpaired or paired t-test as appropriate.

Materials

AMPA was obtained from Research Biochemicals, Inc. (Natick, MA). GYKI 52466 was from Dr Istvan Tarnawa (Budapest, Hungary) and GYKI 53655 was from Dr J. David Leander, Eli Lilly and Co. (Indianapolis, IN). All other drugs, chemicals and antibiotics were from Sigma Chemical Co. (St Louis, MO) and Gibco BRL/Life Technologies (Gaithersburg, MD).

RESULTS

GYKI 52466 *and thiocyanate block of AMPA-evoked currents*

Perfusion of cultured hippocampal neurons with 100 μM AMPA evoked a rapidly activating and inactivating inward current response that decayed to a constant steady-state level within ~30 ms. As illustrated in the traces of Fig. 1A, GYKI 52466 and SCN^- produced a rapid block of the steady-state AMPA response. Fractional block values from a series of similar experiments with various concentrations of the antagonists are plotted in Fig. 1B. There was a concentration-dependent increase in

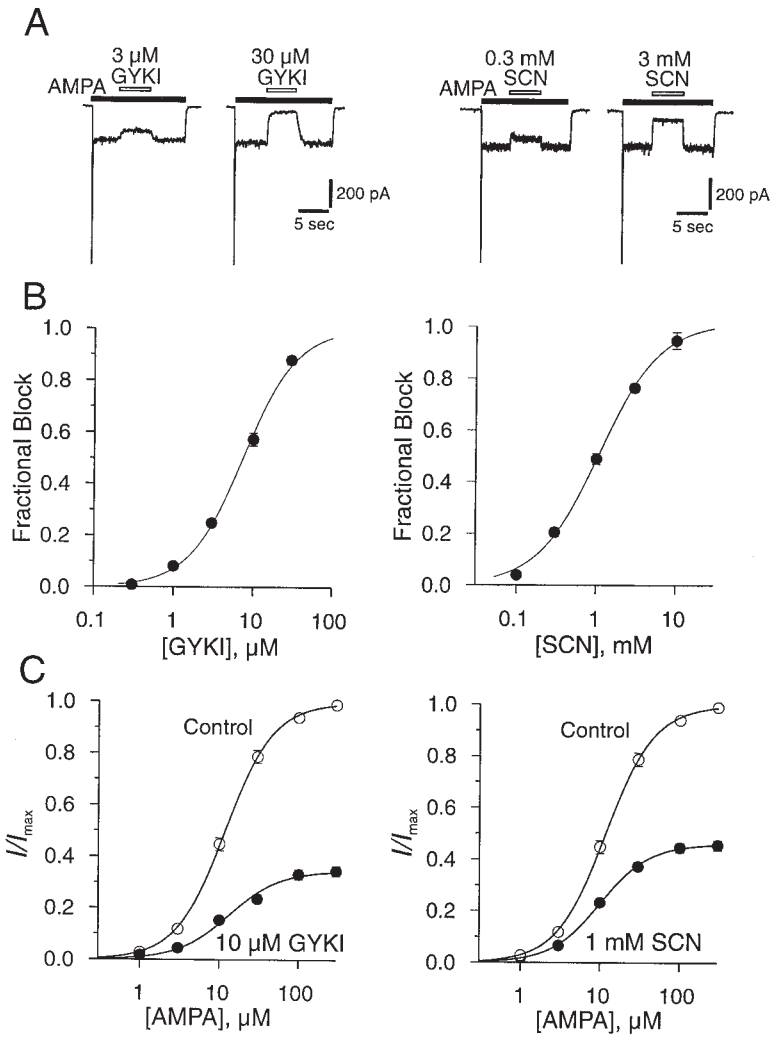

Fig. 1. GYKI 52466 and SCN^- produce a concentration-dependent, non-competitive block of steady-state AMPA receptor current. (A) Representative current traces showing block of 100 μM AMPA-evoked inward currents at −60 mV by two concentrations of the antagonists. (B) Concentration–block isotherms for GYKI 52466 (left) and SCN^- (right). Each point represents the mean ± S.E.M. of data from four to six neurons; where not shown, error bars are smaller than the size of the symbols. (C) Concentration–response curves for steady-state AMPA current in the absence ($n=7$) and presence of 10 μM GYKI 52466 ($n=6$) and 1 mM SCN^- ($n=6$). The currents (I) are normalized to the maximal steady-state AMPA-evoked current (300 μM AMPA) in the absence of antagonist (I_{max}).

fractional block as the concentration of GYKI 52466 was increased within the range 0.3 to 30 μM and SCN^- was increased within the range 0.1 to 10 mM. The IC_{50} values for block by GYKI 52466 and SCN^- were 8.2 ± 0.5 μM ($n=6$) and 1.1 ± 0.1 mM ($n=4$), respectively. These values are similar to those reported previously.[3,5,30] Figure 1C shows AMPA concentration–response curves in the absence and presence of the two antagonists. Consistent with their previously described noncompetitive blocking actions,[3,5] GYKI 52466 and SCN^- produced a reduction in the maximal steady-state response to AMPA without affecting the AMPA concentration-dependence (Fig. 1C). Thus, the maximal AMPA response was reduced to 37 ± 2 and 46 ± 2% of control for 10 μM GYKI 52466 and 1 mM SCN^-, respectively ($P<0.01$), whereas the EC_{50} values were not significantly different from the control value [control, 12 ± 1 μM ($n=6$); GYKI 52466, 16 ± 2 μM ($n=6$); SCN^-, 11 ± 2 μM ($n=6$)].

GYKI 52466 *block and thiocyanate potentiation of kainate-evoked currents*

As illustrated in Fig. 2, GYKI 52466 blocked kainate-evoked currents with similar potency as it blocked steady-state AMPA-evoked current responses. In contrast, SCN^- did not block kainate-evoked current and, at concentrations ≥3 mM, produced a modest potentiation of kainate responses.

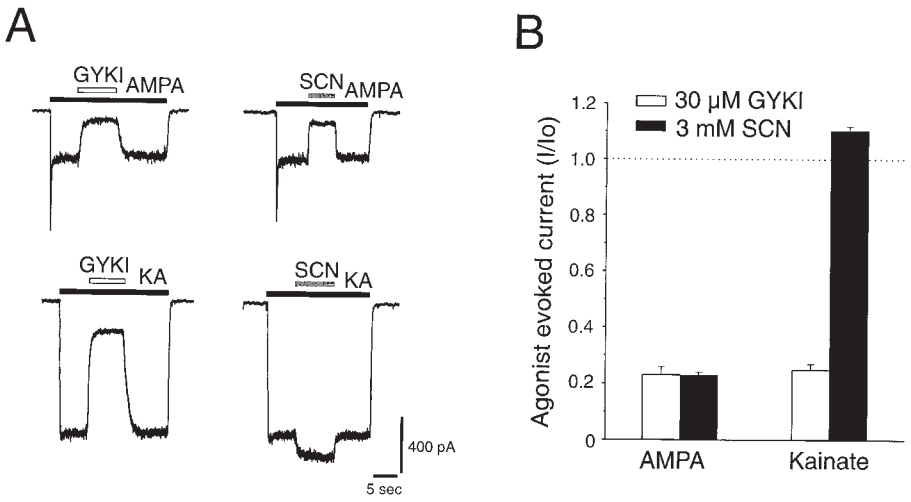

Fig. 2. 2,3-Benzodiazepines and SCN^- differentially affect AMPA- and kainate-evoked responses. (A) Comparison of the effects of 30 µM GYKI 52466 and 3 mM SCN^- on steady-state inward currents evoked by 100 µM AMPA and 100 µM kainate (all traces from the same experiment). (B) Summary of a series of experiments similar to that shown in (A) (n=6–7). Bars indicate the mean ± S.E.M. normalized current (I/I_o) during the application of the antagonists.

For example, in the experiment of Fig. 2A, 30 µm GYKI 52466 and 3 mM SCN^- produced a comparable block of the steady-state response to 100 µM AMPA (top traces). GYKI 52466 also blocked the steady-state response to 100 µM kainate. However, in contrast to its effect on AMPA-evoked current, 3 mM SCN^- enhanced the kainate-evoked response (bottom traces). Data from a series of similar experiments are summarized in Fig. 2B. The mean enhancement of the kainate response produced by 3 mM SCN^- was 10 ± 2% (n=7), a statistically significant increase (P<0.05). A higher concentration of SCN^- (10 mM) produced an even larger enhancement (32 ± 2%; n=6) of the kainate response (data not shown).

Effects of thiocyanate on N-*methyl*-D-*aspartate- and GABA-evoked currents*

GYKI 52466 is a selective antagonist of non-NMDA receptors having little effect, even at high concentrations, on currents evoked by NMDA or GABA.[5] We examined whether SCN^- had a similarly selective action by testing the effects of 10 mM SCN^- on inward currents evoked by 10 µM NMDA (in the presence of 10 µM glycine and absence of Mg^{2+}) and 5 µM GABA. SCN^- failed to block NMDA- and GABA-evoked currents; moreover, as was the case for kainate-evoked currents, both NMDA- and GABA-evoked currents were significantly (P<0.01) enhanced in the presence of 10 mM SCN^- [32 ± 3% (n=7) and 53 ± 6% (n=5), respectively].

Comparison of the effects of GYKI 52466 *and thiocyanate on AMPA receptor desensitization*

Experiments were conducted comparing the effects of GYKI 52466 and SCN^- on the desensitization of currents evoked by 1 mM AMPA in outside-out excised patches. Because of the high density of AMPA receptors in the patches, ensemble currents were recorded that closely mimicked whole-cell AMPA-evoked currents. However, since solution exchange in the patch recordings is more rapid, it was possible for the time-course of desensitization following onset of the AMPA perfusion to be more accurately assessed. The time-course of AMPA receptor current desensitization in patch recordings under control conditions was well fit by single exponentials with time constants ranging from 4 to 13 ms (mean ± S.E.M: 8.4 ± 0.7 ms; n=15). In confirmation of a previous report,[5] 30 µM GYKI 52466 did not affect the desensitization time constant of AMPA-evoked current responses and produced a similar block of peak (patch recordings) and steady-state (whole-cell recordings) responses to AMPA (Fig. 3A, C, D). In contrast, 3 mM SCN^- produced a significant decrease in the time constant for desensitization of the excised patch AMPA responses (Fig. 3B, C) and produced only minimal block of the peak AMPA response (Fig. 3D).

To further compare the effects of GYKI 52466 and SCN^- on AMPA receptor desensitization we examined the actions of these compounds on recovery from desensitization in outside-out patches using a paired-pulse paradigm in which two 50 ms applications of AMPA were separated by time intervals ranging from 20 to 1000 ms. An example of such an experiment is illustrated in the top traces of Fig. 4A. The ratios of the amplitudes of the second and first responses (P_2/P_1) are plotted against the interpulse interval in Fig. 4B. The control recovery ratios were well fit by a single exponential with a time constant of 247 ms. As demonstrated by the traces in the middle panel of Fig. 4A, 30 µM GYKI 52466 had little effect

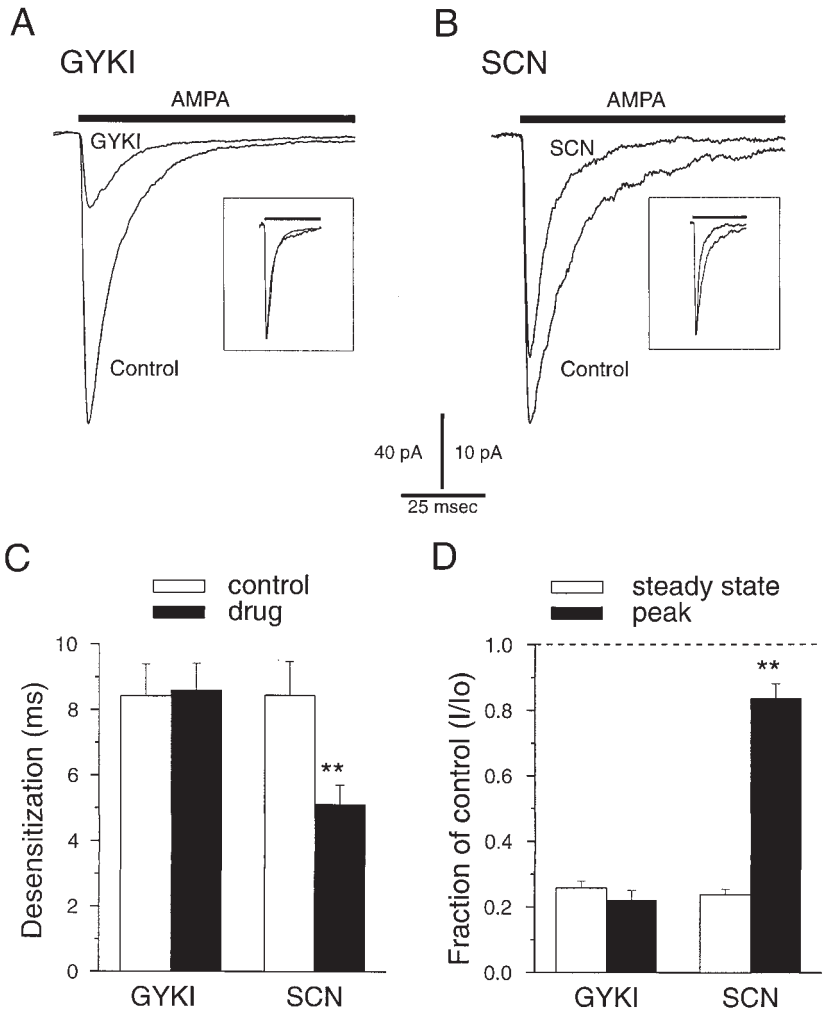

Fig. 3. Contrasting effects of GYKI 52466 and SCN^- on AMPA receptor desensitization. Except as noted, the data are from outside-out patch recordings. The traces in (A) and (B) represent the initial current transient evoked by 1 mM AMPA in the absence and presence of 30 μM GYKI 52466 and 3 mM SCN^-; each trace represents the averaged current evoked by five to 10 consecutive agonist applications. The insets show the traces scaled to the same amplitude. (C) Time constant values (mean ± S.E.M.) from the best fit single exponentials to data similar to that shown in A and B. GYKI 52466 (n=7) had no effect on time constant of desensitization, whereas SCN^- (n=8) speeded desensitization (P<0.01). (D) Comparison of effects of 30 μM GYKI 52466 and 3 mM SCN^- on the steady-state current evoked by 100 μM AMPA in whole-cell recordings and on the peak amplitudes of the transients evoked by 1 mM AMPA in outside-out patches (n=4–7).

on the time-course of recovery from desensitization. In contrast, 10 mM SCN^- produced a marked slowing of the time-course of recovery (Fig. 4A, bottom panel). The time constants obtained from the best fits to the recovery curves for 30 μM GYKI 52466 and 10 mM SCN^- (Fig. 4B) were 255 and 934 ms, respectively. A lower concentration of SCN^- (3 mM) produced an intermediate slowing of recovery from desensitization (time constant, 568 ms; data not shown).

Interactions with cyclothiazide

Cyclothiazide has been reported to prevent the rapid desensitization of AMPA receptor currents.[20,28,30] However, there may be differences among neurons in the extent to which this effect occurs[12] relating to variations in the expression of the *flip/flop* splice variants.[19] As demonstrated in Fig. 5, cultured hippocampal neurons also showed wide variability in their sensitivity to cyclothiazide. Thus, kainate responses in some neurons were only modestly potentiated by cyclothiazide (Fig. 5A) whereas in other neurons the magnitude of potentiation was larger (Fig. 5B). In neurons that expressed smaller degrees of kainate potentiation, cyclothiazide less effectively reduced desensitization of AMPA-evoked responses. Neurons that were relatively insensitive to cyclothiazide also showed more rapidly and completely desensitizing responses to AMPA and a larger

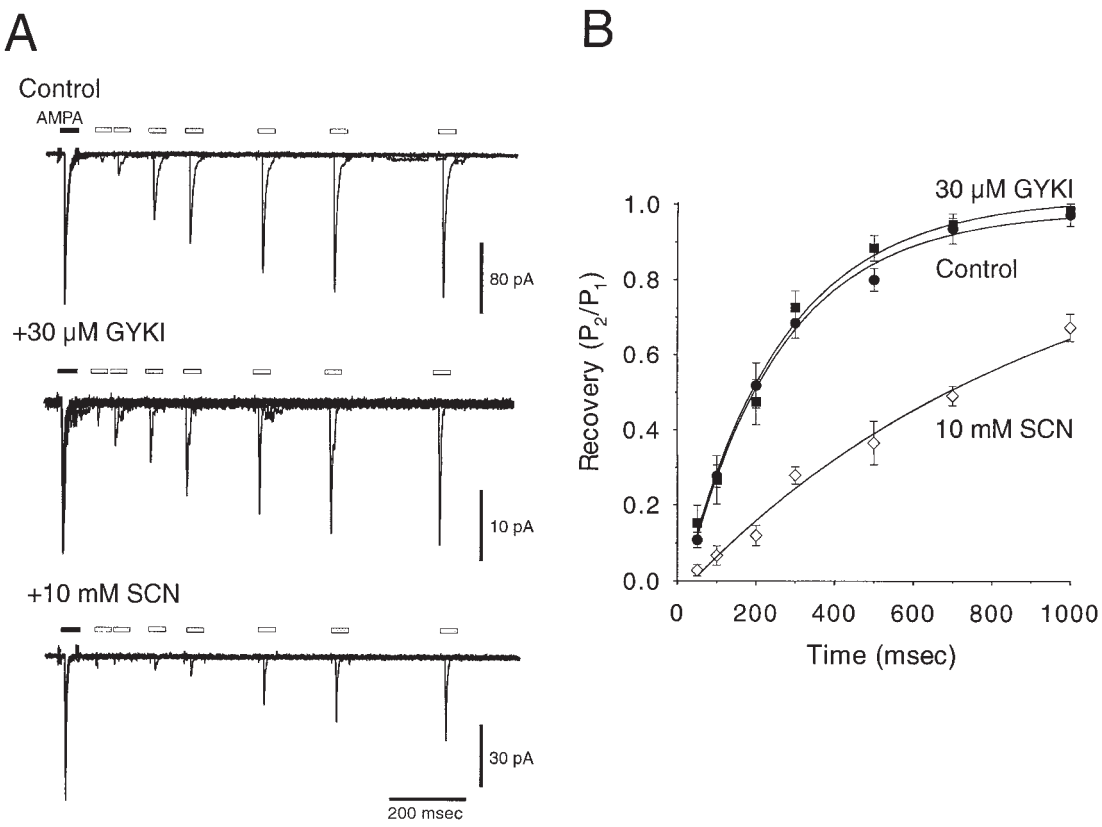

Fig. 4. Effects of GYKI 52466 and SCN^- on recovery from desensitization. (A) Superimposed current responses obtained in outside-out patch recordings exposed to paired 50 ms applications of 1 mM AMPA. The dark and light bars indicate the periods of application of the first and second pulses, respectively. The interpulse interval was varied from 20 to 1000 ms. Experiments were conducted in three separate patches in the absence (top panel) or presence of 30 μM GYKI 52466 (middle panel) or 10 mM SCN^- (bottom panel). (B) Recovery curves from experiments similar to those shown in A. Each data point represents the mean ± S.E.M. of the ratio P_2/P_1 where P_1 and P_2 are the peak current amplitudes of the first and second pulse, respectively (n = 7–10). Data points are fit to single exponential functions with time constants given in the text.

amplitude steady-state kainate current response than steady-state AMPA response (Fig. 5A) as expected of AMPA receptors composed mainly of subunits containing the *flop* splice cassette.[19] Overall, the extent to which 30 μM cyclothiazide potentiated kainate-evoked whole cell responses varied from 1.3- to 10-fold.

If GYKI 52466 or SCN^- act at the same site as cyclothiazide there should be a correlation between the sensitivity to block by the antagonist and the sensitivity to cyclothiazide. This was addressed in experiments in which, in the same cell, the magnitude of the potentiation of the whole-cell kainate response by cyclothiazide was compared with the degree of block of the steady-state AMPA-evoked inward current response by GYKI 52466 and by SCN^-. As shown in Fig. 6A, there was no correlation between the degree of potentiation by 30 μM cyclothiazide and the block of 1 mM AMPA currents by 10 μM GYKI 52466 (r = 0.1, $d.f.$ = 18). In contrast, there was a highly significant correlation between SCN^- block and cyclothiazide potentiation in this same group of cells (r = 0.8, $d.f.$ = 18; P < 0.01, Fig. 6B). As an alternative way of expressing these data, cells were selected that showed low (<two-fold; n = 7) and high (>four-fold; n = 7) degrees of potentiation by cyclothiazide. As shown in Fig. 6C, the block by GYKI 52466 in these two groups was similar. In contrast, SCN^- produced a significantly greater block in the high cyclothiazide potentiation group (P < 0.01).

The correlation between SCN^- block and cyclothiazide potentiation raised the possibility that the two allosteric modulators may act at common (overlapping) regions of the AMPA receptor. To examine this possibility more directly, we carried out experiments to determine if cyclothiazide can affect the blocking potency of SCN^-. We reasoned that if cyclothiazide and SCN^- bind to overlapping regions of the AMPA receptor, then cyclothiazide should reduce the blocking potency of SCN^- at any single concentration and cause a rightward displacement of the SCN^- concentration–block curve. As illustrated in the experiment of Fig. 7A, these predictions were borne out. Thus, 3 mM SCN^- produced an almost

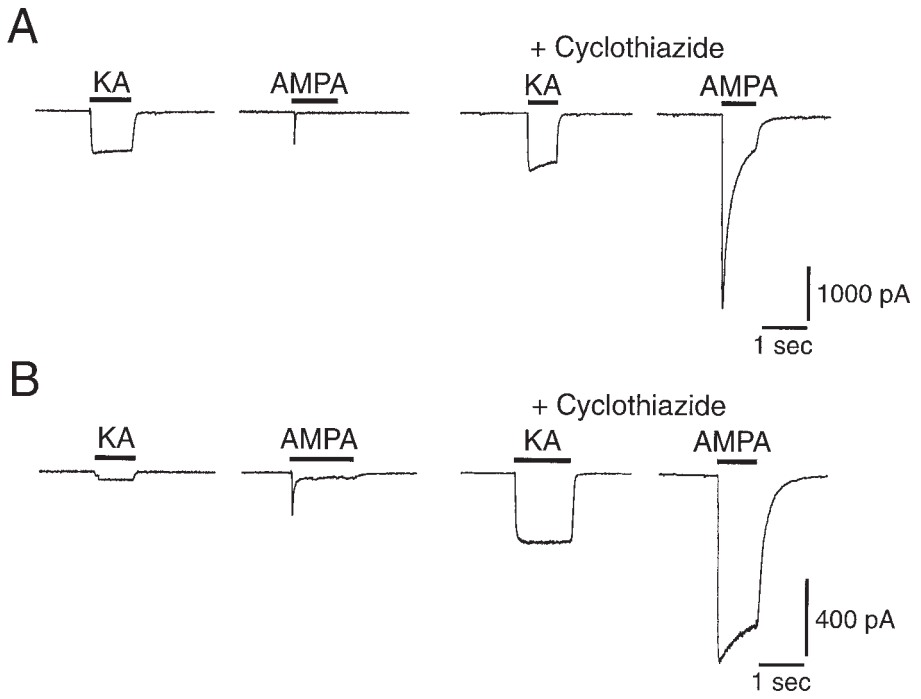

Fig. 5. Variable potentiation of AMPA receptor responses by cyclothiazide. In the neuron represented in (A), 30 μM cyclothiazide produces relatively less potentiation of the responses to 100 μM kainate and 1 mM AMPA than in the neuron of (B).

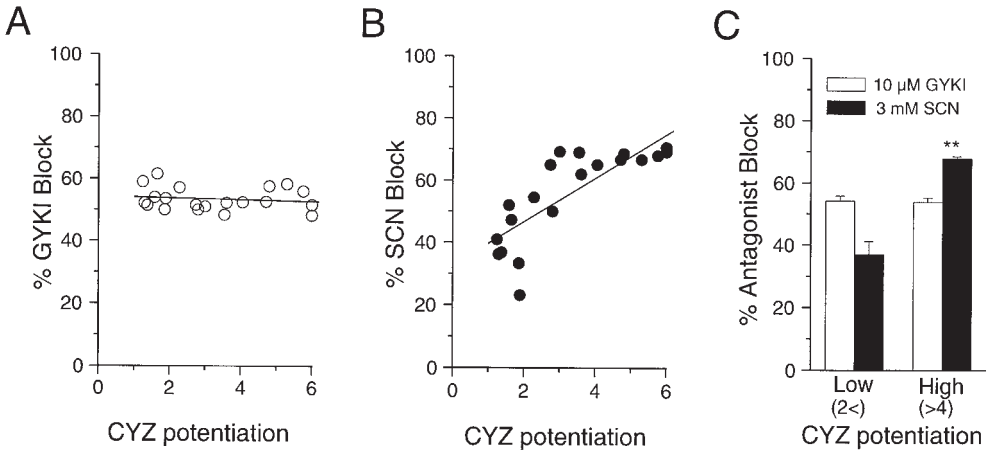

Fig. 6. SCN^-, but not GYKI 52466, block correlates with cyclothiazide sensitivity. Comparison of the degree of potentiation of 100 μM kainate-evoked currents by 30 μM cyclothiazide with degree of block of 1 mM AMPA-evoked currents in the same cell by 10 μM GYKI 52466 (A) and 3 mM SCN^- (B). (C) Summary of data from A and B showing the degree of GYKI 52466 and SCN^- block of AMPA currents in cells that exhibited low (<two-fold) or high (>four-fold) potentiation of kainate currents by cyclothiazide. **Significantly different from ''low'' group ($P<0.01$); the mean values for the two GYKI 52466 groups were not significantly different.

complete block of the steady-state AMPA current, but had markedly reduced potency in the presence of 30 μM cyclothiazide. Moreover, as shown in Fig. 7B, 10 μM cyclothiazide produced a rightward shift in the concentration–response curve for SCN^- block of steady-state AMPA responses. The IC_{50} values obtained from logistic fits to the fractional block values in the absence and presence of cyclothiazide were 1.1 (n_H, 1.1) and 8.5 mM (n_H, 0.9), respectively.

If the sites of action of SCN^- and cyclothiazide overlap, then the rate of onset of SCN^- block in the presence of cyclothiazide should be limited by (equal to or slower than) the rate at which cyclothiazide dissociates. To determine if the rates are compatible

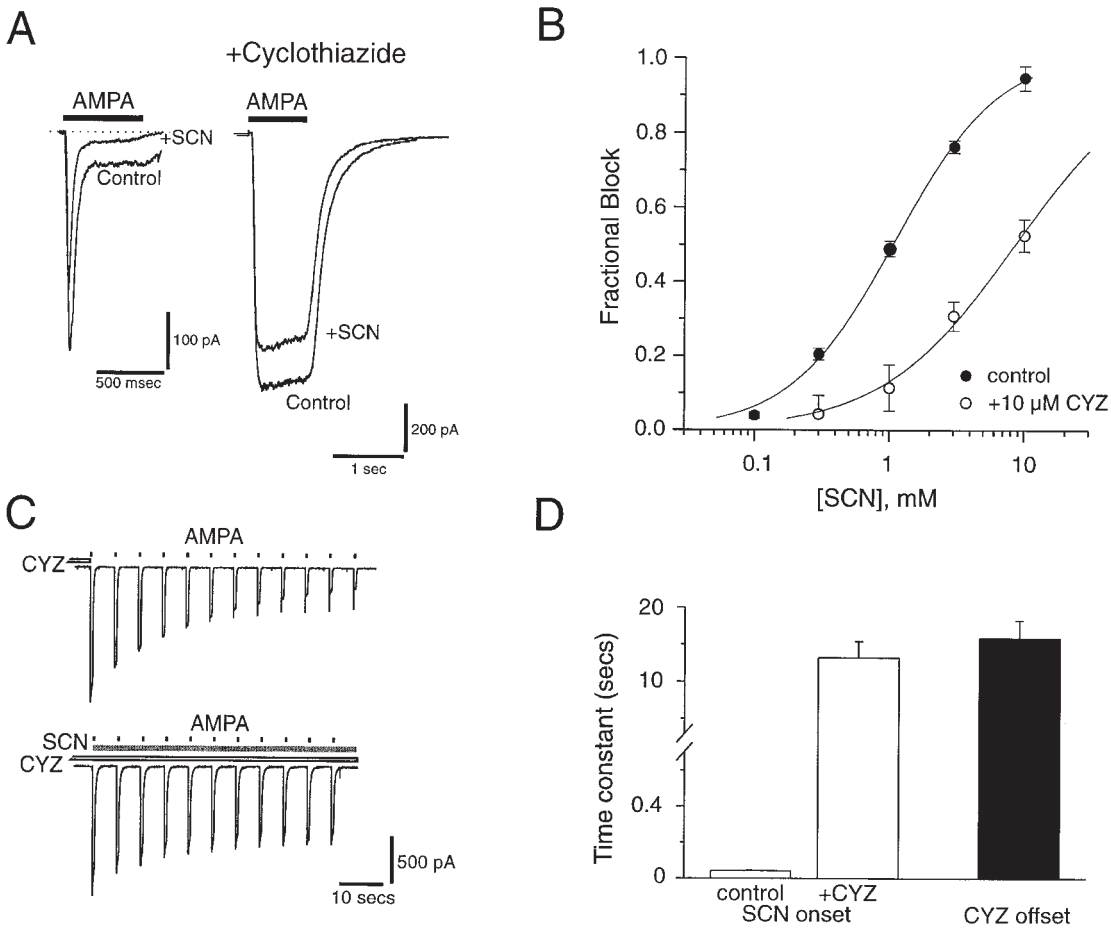

Fig. 7. Evidence supporting a competitive interaction between SCN^- and cyclothiazide. (A) Whole-cell recordings demonstrating that the block of the 1 mM AMPA current by 3 mM SCN^- is reduced in the presence of 30 μM cyclothiazide (all traces from the same cell). (B) Concentration–block curves for SCN^- in the absence (●) and presence (○) of 10 μM cyclothiazide. The data points represent the mean ± S.E.M. values for block of the steady-state currents in three to eight cells. (C) Comparison of the time-course for recovery from 10 μM cyclothiazide potentiation of 1 mM AMPA currents (top panel) with the time-course for the onset of 3 mM SCN^- block in the continuous presence of cyclothiazide (bottom panel). AMPA was applied in 500 ms duration pulses separated by 5 s. (D) Comparison of the time constant for the onset of block of the steady-state AMPA current by 3 mM SCN^- in the absence (control) and presence of 10 μM cyclothiazide (+CYZ) with the time constant for cyclothiazide dissociation. The data for SCN^- onset in the presence of cyclothiazide and for cyclothiazide offset were from six experiments similar to those shown in C. The time constant values were determined from single exponential fits to the peak current amplitudes at each time point; there was no significant difference between the mean time constant values ($P<0.05$). The time constants for SCN^- block in the absence of cyclothiazide were measured by fitting the current trajectory in experiments similar to that shown in Fig. 1A ($n=4$).

with this model, we carried out experiments in which the time constants were measured by assessing the level of potentiation or block of the currents evoked by repeated brief (500 ms) 1 mM AMPA pulses. The traces in the top panel of Fig. 7C illustrate such an experiment whose objective was to measure the time constant of recovery from 10 μM cyclothiazide; the bottom panel shows a corresponding experiment to determine the onset of SCN^- block in the presence of 10 μM cyclothiazide. The time constants were determined by fitting the peak current amplitudes to single exponential functions. In a series of similar experiments, the mean time constant value for onset of SCN^- block in the presence of cyclothiazide was roughly equal to the decay of cyclothiazide potentiation and much slower than the onset of SCN^- block in the absence of cyclothiazide (Fig. 7D).

Our observations (i) that GYKI 52466 failed to affect the desensitization of AMPA-evoked currents, and (ii) that the magnitude of GYKI 52466 block was not correlated with the magnitude of cyclothiazide potentiation indicated that 2,3-benzodiazepines and cyclothiazide do not act at the same site. Nevertheless, it has previously been demonstrated that cyclothiazide can reverse the blocking action of 2,3-benzodiazepines, which raised the possibility of a

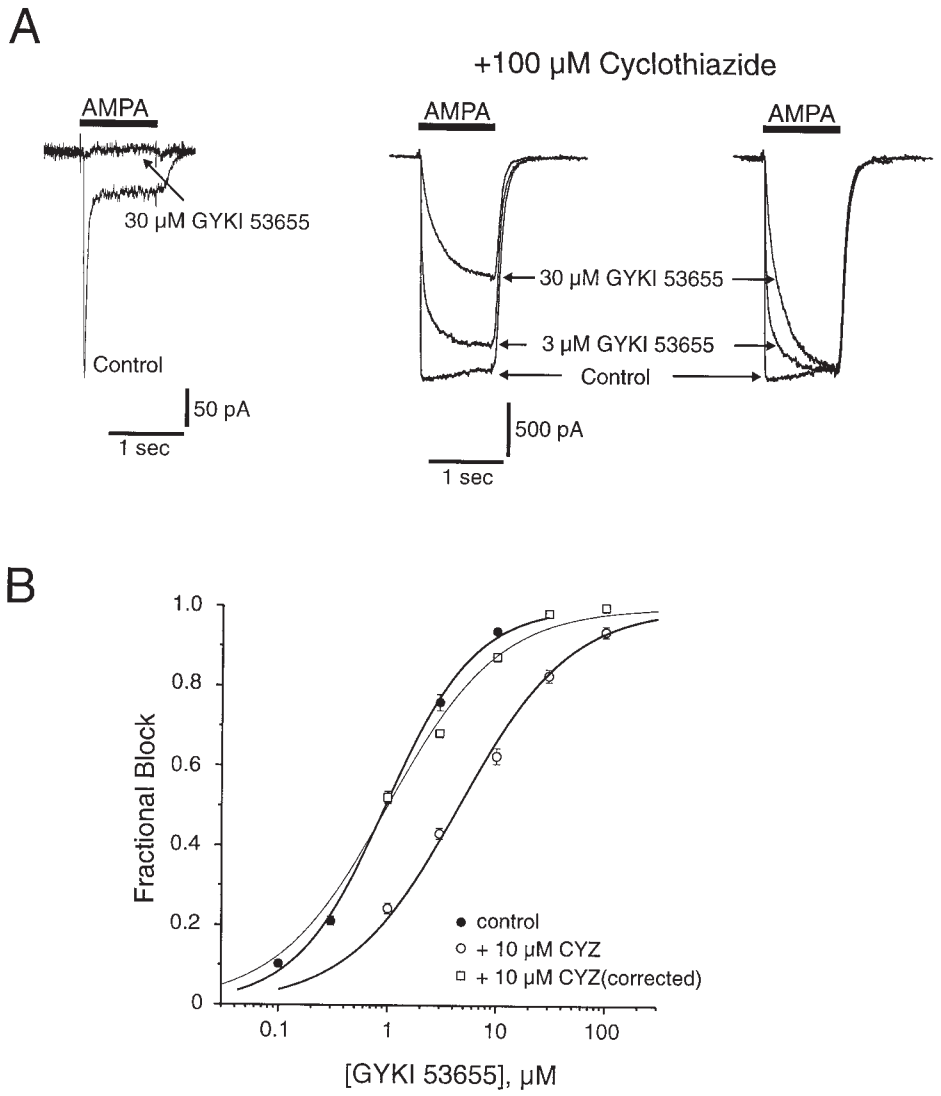

Fig. 8. Evidence against a competitive interaction between GYKI 53655 and cyclothiazide. (A) Block of 1 mM AMPA responses by GYKI 53655 in the absence (left) and presence (right) of 30 μM cyclothiazide. (B) Concentration–response curves for GYKI 53655 block of the steady-state AMPA-evoked current in the absence (●) and presence (○) of 10 μM cyclothiazide. Each point represents the mean ± S.E.M. of the fractional block values from three to six cells. Mean values for block of the initial fast component of the currents recorded in the presence of cyclothiazide are also shown (□; see text). The smooth curves are logistic fits to the mean data values.

competitive interaction.[16,30] In order to address this possibility further, we carried out a series of experiments with GYKI 53655 that were similar to those described above for SCN^-. As with SCN^-, GYKI 53655 block of AMPA currents was reduced in the presence of cyclothiazide (Fig. 8A). In addition, 10 μM cyclothiazide caused a rightward shift in the concentration–response curve for GYKI 53655 block of 100 μM AMPA currents (Fig. 8B; IC_{50} values, 1.0 and 4.5 μM in the absence and presence of cyclothiazide, respectively). However, as seen in Fig. 8A, there was a dramatic alteration in the shape of the AMPA-evoked current response in the presence of cyclothiazide and GYKI 53655. In the presence of both drugs, the AMPA-evoked current did not rapidly activate and inactivate as under control conditions but instead rose slowly to a plateau during the 1 s AMPA application. The AMPA-evoked current response in the presence cyclothiazide alone exhibited a fast rise time that was adequately fit by a single exponential function (time constant, 19 ± 2 ms; n = 11). In contrast, in the presence of cyclothiazide and GYKI 53655, the current trajectory had two components: an initial fast component with time constant similar to that obtained with cyclothiazide alone and an additional late slow component. As the concentration of GYKI 53655 was increased, the relative amplitude of the fast component decreased and the

Table 1. Fast and slow components of GYKI 53655 block of AMPA-evoked currents in the presence of cyclothiazide

GYKI 53655 (μM)	*n*	τ_f (ms)	A_f (%)	τ_s (ms)	A_s (%)
1	4	23 ± 1	70 ± 3	159 ± 42	30 ± 3
3	5	18 ± 2	61 ± 1	257 ± 21	39 ± 1
10	6	19 ± 2	36 ± 2	531 ± 81	64 ± 2
30	4	17 ± 2	9 ± 2	595 ± 49	91 ± 2
100	6	N.D.	0	564 ± 64	100

Fast time constant (τ_f) values were determined by fitting the onset of 1 mM AMPA responses in the presence of 10 μM cyclothiazide to a single exponential function. Slow time constant (τ_s) values and relative amplitudes of the fast (A_f) and slow (A_s) components were determined by fitting the onset of 1 mM AMPA responses in the presence of 10 μM cyclothiazide and the indicated concentration of GYKI 53655 to the biexponential function given in the text (constraining the τ_f parameter to the value obtained with cyclothiazide alone in the same cell). The τ_s values for 1 and 3 μM GYKI 53655 are unreliable because the overall degree of block and the relative amplitude of the slow component were small. N.D. not determined.

relative amplitude of the slow component increased (Table 1). These observations are consistent with a model in which AMPA decreases the binding affinity of GYKI 53655. Thus, when AMPA is applied, GYKI 53655 unbinds slowly from the receptor and a reduced level of steady-state block is eventually achieved. Accordingly, the fast component of the response reflects the onset of the AMPA response prior to the slow unbinding of GYKI 53655 (with the level of block reflecting the higher affinity for GYKI 53655 in the absence of AMPA) and the slower component reflects the slow unbinding of GYKI 53655 as the new steady-state level of block is achieved. At high GYKI 53655 concentrations (such as in Fig. 8A, right), there was nearly complete block at the onset of the AMPA perfusion. Then, as GYKI 53655 dissociates and the receptors are gradually unblocked, a slow rise in the current occurs. To estimate the level of block at the onset of the AMPA perfusion, we fit the trajectory of the current responses to a two exponential function $A_f[1-\exp(-t/\tau_f)]+A_s[1-\exp(-t/\tau_s)]$ where A_f and A_s are the amplitudes of the fast and slow components, respectively, and τ_f and τ_s are the corresponding time constants. In the analysis of data for each individual cell, τ_f was constrained to the time constant of the onset of the AMPA response in the presence of cyclothiazide alone (Table 1). The fractional block of the A_f values (''corrected'' fractional block) are plotted in Fig. 8B. The IC_{50} value obtained from a logistic fit to these data was 1 μM (n_H, 0.9) which is identical to the IC_{50} value for GYKI 53655 block of AMPA currents in the absence of cyclothiazide (1 μM) (Fig. 8B). We have previously shown that 2,3-benzodiazepines are able to bind and block AMPA receptors in the absence of agonist (i.e. AMPA).[5] The present data indicate that the GYKI 53655 binding affinity in the absence of AMPA is unaffected by cyclothiazide. However, when AMPA is applied in the presence of cyclothiazide there is a reduction in affinity for the antagonist that does not occur in the absence of cyclothiazide. Because of this reduction in affinity, there is a rightward shift in the concentration–response curve for GYKI 53655 steady-state block (Fig. 8B). However, the initial level of block is unaffected by cyclothiazide indicating that cyclothiazide does not compete with GYKI 53655 for binding.

Finally, as described for SCN^- above, if GYKI 53655 and cyclothiazide were to act at the same site, then the rate of onset of GYKI 53655 block should be limited by the dissociation rate of cyclothiazide. Because AMPA responses had a slow onset in the presence of GYKI 53655 and cyclothiazide, it was not possible to assess the development of block according to the protocol of Fig. 7C. Instead, during the continuous presence of 100 μM cyclothiazide, we determined the level of block with a 500 ms pulse of 1 mM AMPA applied at various intervals (from 10 ms to 8 s) after the onset of perfusion with 30 μM GYKI 53655 (Fig. 9A). The amplitudes of the fast component (A_f) were determined as described above and these values were fit to single exponential functions using the delay interval between onset of the GYKI 53655 perfusion and the onset of the pulse of AMPA as the independent variable. As illustrated in the bar chart of Fig. 9B, the time constant for onset of GYKI 53655 block in the presence of cyclothiazide is similar to the time constant for the onset of GYKI 53655 block of the steady-state AMPA-evoked currents in the absence of cyclothiazide (from Donevan *et al.*[7]) but more than 250-fold faster than the dissociation (recovery) rate for cyclothiazide. These kinetic observations further confirm that the reduction of GYKI 53655 block produced by cyclothiazide does not result from a competitive interaction of the drugs at a common binding site.

Thiocyanate block of recombinant AMPA receptor subunits expressed in Xenopus *oocytes*

Because *flip* AMPA receptor splice variants are more strongly potentiated by cyclothiazide than the *flop* forms, it has been proposed that the *flip/flop* module directly contributes to the binding site for cyclothiazide.[9,19] If SCN^- and cyclothiazide bind to common receptor domains, SCN^- may be expected to block the *flip* forms more strongly than it blocks

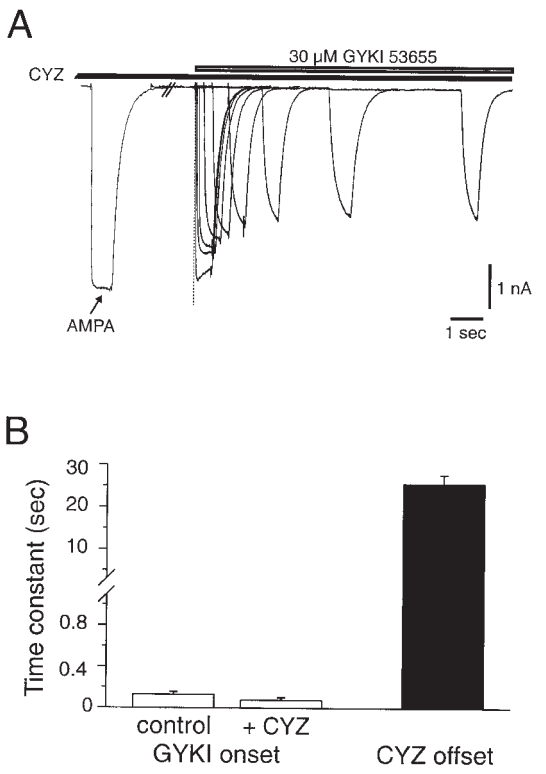

Fig. 9. Comparison of the kinetics for onset of block by GYKI 53655 and dissociation of cyclothiazide. (A) Time-course for onset of 30 μM GYKI 53655 block of 1 mM AMPA currents in the presence of 100 μM cyclothiazide. Traces from 10 separate 500 ms AMPA applications are shown. The control AMPA response in the presence of cyclothiazide is marked with an arrow; the superimposed traces show AMPA responses with increasing intervals (10, 50, 100, 250, 500 ms, 1, 2, 4, 8 s) between onset of the GYKI 53655 perfusion and the AMPA application. (B) Time constants for the onset of 30 μM GYKI 53655 block of AMPA currents in the absence ("control"; taken from Donevan *et al.*[7]) and presence of 100 μM cyclothiazide with the time constant for recovery from cyclothiazide potentiation of AMPA currents. Each bar represents the mean ± S.E.M. of data from four to six cells. The time constant for onset of GYKI 53655 block in the presence of cyclothiazide was determined in experiments similar to that illustrated in C where the initial level of block was determined as described in the text. The time constant for recovery from cyclothiazide potentiation was determined as in Fig. 7C.

flop forms. This was examined in recordings of *flip* and *flop* AMPA receptor subunits expressed in *Xenopus* oocytes. Figure 10A illustrates the concentration-dependent inhibitory effect of SCN^- on AMPA-evoked currents in oocytes injected with various *flip* subunit cRNAs. Among the limited survey of subunit and subunit combinations that was carried out, $GluR1_{flip}/GluR2_{flip}$ was most sensitive to SCN^- block. As shown by the concentration–block curves of Fig. 10B, all *flip*-containing splice variants were more sensitive to SCN^- than $GluR1_{flop}$. The IC_{50} values obtained from logistic fits to the mean fractional block values for $GluR1_{flip}/GluR2_{flip}$ and $GluR3_{flip}$ were 0.9 ± 0.1 and 2.6 ± 0.5 mM, respectively. For $GluR1_{flip}$ and $GluR1_{flop}$, even the highest concentration of SCN^- failed to reduce the AMPA-evoked current by 50% so that IC_{50} values could not be reliably determined; estimates of the IC_{50} values by extrapolation were 14 and 77 mM, respectively.

DISCUSSION

2,3-Benzodiazepines[5,30] and SCN^-,[3] block AMPA receptor currents in a noncompetitive fashion, indicating that they act at allosteric regulatory site(s) on the AMPA receptor that are distinct from the AMPA recognition site. In the present study we sought to determine whether the two blocking agents interact with the same or distinct sites on AMPA receptors. A principal conclusion from our results is that the two classes of antagonist act at distinct allosteric sites and by different mechanisms. Our data further indicate that SCN^- may interact with a region of the AMPA receptor that overlaps the positive modulatory site of cyclothiazide, and supports the concept of Arai *et al.*[1] that the block produced by SCN^- occurs as a result of an acceleration and enhancement of desensitization. In contrast, the block produced by the 2,3-benzodiazepines occurs at a site that is distinct from the cyclothiazide site and does not involve enhancement of desensitization.

Comparison of effects on AMPA- and kainate-evoked currents

Initial support for these conclusions derives from studies comparing the effects of the antagonists on AMPA- and kainate-evoked currents. 2,3-Benzodiazepines blocked steady-state AMPA- and kainate-evoked currents with similar potency, whereas SCN^- blocked only AMPA-evoked currents and at high concentrations actually enhanced kainate-evoked currents. These latter observations confirm recently reported results with recombinant AMPA receptor subunits expressed in *Xenopus* oocytes where SCN^- also potentiated kainate-activated responses.[8] The bulk of the current evoked by kainate in cultured hippocampal neurons is carried by AMPA receptors.[11,22] AMPA receptors gated by AMPA rapidly desensitize whereas AMPA receptors gated by kainate do not exhibit desensitization. Cyclothiazide can potentiate kainate responses, however, and it is therefore likely that kainate responses as recorded under the present experimental conditions are fully desensitized (some degree of desensitization may be observed with faster perfusion methods[23]). Since SCN^- acts to enhance conversion of AMPA receptors to the desensitized state,[1] it is perhaps not surprising that SCN^- does not block kainate-evoked currents inasmuch as kainate responses are already fully desensitized. It is not apparent, however, why SCN^- enhances the amplitude of kainate-evoked current responses. Since SCN^- also

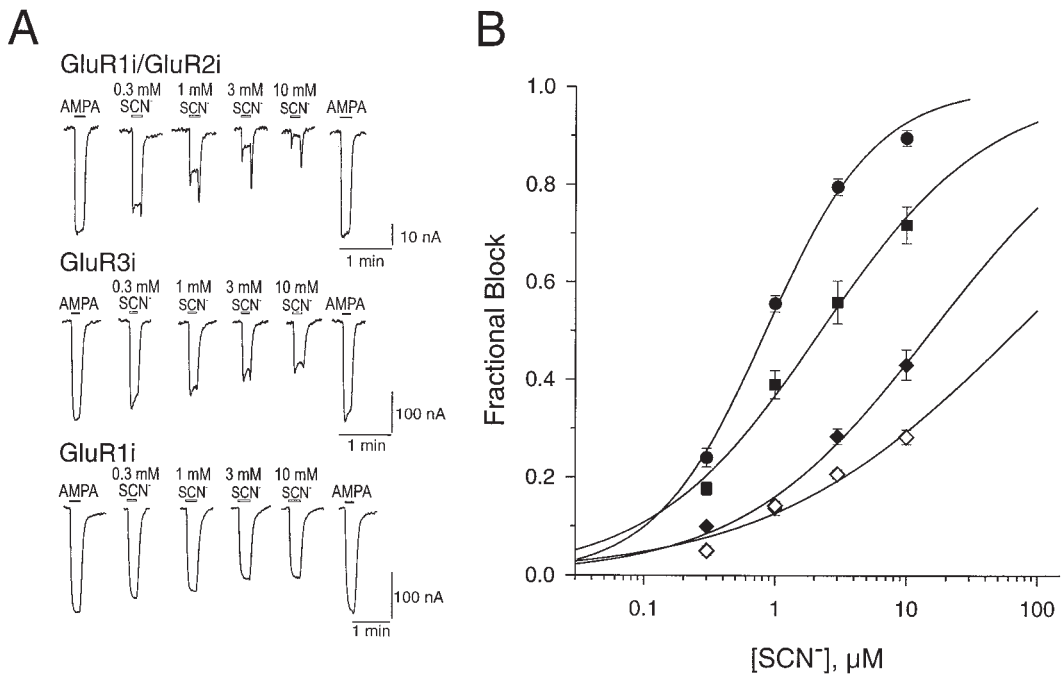

Fig. 10. SCN^- block of AMPA receptor currents in *Xenopus* oocytes injected with *flip* and *flop* subunit cRNAs. (A) Sample current traces demonstrating concentration-dependent SCN^- inhibition of AMPA-evoked currents in oocytes injected with $GluR1_{flip}/GluR2_{flip}$ (1:4) (top), $GluR3_{flip}$ (middle) and $GluR1_{flip}$ (bottom). SCN^- at the indicated concentrations was co-applied with 100 μM AMPA; control and recovery responses to AMPA are shown. (B) Concentration–block curves for a series of experiments similar to those in A. Each point represents the mean ± S.E.M. of fractional block values from experiments with 5–6 oocytes. ●, $GluR1_{flip}/GluR2_{flip}$; ■, $GluR3_{flip}$; ◆, $GluR1_{flip}$; ◇, $GluR1_{flop}$.

produced a potentiation of NMDA and GABA-evoked current responses, it is possible that the potentiation may be a non-specific effect of the SCN^- anion (possibly related to its chaotropic properties). Indeed, if SCN^- also enhanced AMPA-evoked responses, this effect would be masked by the strong effect of the anion on desensitization. The observation that GYKI 52466 is equally effective as an antagonist of AMPA- and kainate-gated AMPA receptor currents suggests that the 2,3-benzodiazepine does not act like SCN^- to enhance desensitization.

Effects on kinetic properties of AMPA receptor currents

Differences in the effects of GYKI 52466 and SCN^- on the kinetic properties of AMPA receptor currents serve to further contrast the two antagonists. Thus, GYKI 52466 was equally effective at blocking peak and steady-state AMPA evoked-currents and did not affect the time-course of the currents. In contrast, SCN^- had little effect on peak AMPA receptor currents, but caused a marked acceleration in the speed and extent of desensitization. Furthermore, while GYKI 52466 had no effect on the time-course of the onset and recovery from desensitization, SCN^- produced a significant speeding of the onset of desensitization and a slowing of recovery from desensitization. These observations further confirmed that SCN^- and the 2,3-benzodiazepines have different mechanisms of action and that SCN^-, but not GYKI 52466, block the AMPA receptor by enhancing desensitization.

Studies with cyclothiazide

Additional support for the conclusion that the 2,3-benzodiazepines do not block AMPA receptors by enhancing desensitization derives from studies with cyclothiazide. Cyclothiazide has previously been reported to reverse GYKI 52466 block of AMPA receptor responses. It has been proposed that there is a competitive (but functionally inverse) interaction between cyclothiazide and the 2,3-benzodiazepines, so that cyclothiazide enhances AMPA receptor currents by inhibiting desensitization and the 2,3-benzodiazepines block AMPA receptor currents by promoting desensitization via actions at the same modulatory site.[16,30] Indeed, we also observed that cyclothiazide produced an apparent reversal of GYKI 53655 block of AMPA-evoked currents and, moreover, appeared to induce a rightward shift in the GYKI 53655 concentration–block curve (Fig. 8A, B). However, there was a dramatic alteration in the shape of AMPA receptor currents recorded in the presence of cyclothiazide and GYKI 53655. The simplest interpretation of this kinetic effect is that, in

the presence of cyclothiazide, agonist gating of the AMPA receptor elicits a slow decrease in the binding affinity for 2,3-benzodiazepines. Only when the level of block is measured after GYKI 53655 dissociates from the receptor, does it appear that cyclothiazide reverses the block. When we estimated the GYKI 53655 block at the onset of the AMPA perfusion in the presence of cyclothiazide, we failed to observe a reduced level of block from that obtained in the absence of cyclothiazide. This represents strong evidence against a competitive interaction between the 2,3-benzodiazepines and cyclothiazide (i.e. 2,3-benzodiazepines and cyclothiazide act at distinct allosteric regulatory sites), and is consistent with the conclusions of several recent studies.[4,9,25,29] Moreover, the observation that the rate of GYKI 53655 block is faster than the rate of cyclothiazide dissociation is incompatible with the possibility that the two allosteric modulators act at overlapping sites. In contrast, the blocking action of SCN^- was reduced in an apparent competitive fashion by cyclothiazide. In addition, the rate of SCN^- block in the presence of cyclothiazide appeared to be limited by cyclothiazide dissociation. These results are consistent with the possibility that SCN^- and cyclothiazide act at a common or closely related site, but have functionally inverse effects.

Further analysis of the data with cyclothiazide provided additional evidence supporting the concepts proposed above. There was cell-to-cell variability in the extent to which cyclothiazide potentiated AMPA- and kainate-evoked current responses in the hippocampal cultures. Livsey *et al*.[12] have shown a similar heterogeneity in the cyclothiazide sensitivity of AMPA receptor responses in patches obtained from spiny neurons in rat hippocampal slices. These differences in cyclothiazide sensitivity may relate to differences in the expression of alternatively spliced forms of the AMPA receptor subunits as Partin *et al*.[19] have demonstrated that AMPA receptors with the *flip* module are more strongly potentiated by cyclothiazide than those containing the *flop* module. If 2,3-benzodiazepines act at the cyclothiazide binding site then similar heterogeneity should exist in the extent to which AMPA receptor responses are blocked. This was not the case, however, as GYKI 52466 was uniformly effective as an antagonist irrespective of the degree to which the AMPA receptor currents were potentiated by cyclothiazide (over a two- to 10-fold range). Thus, all of the available evidence is consistent with the conclusion that the 2,3-benzodiazepines do not exert their blocking action via the site where cyclothiazide acts to inhibit AMPA receptor desensitization. Furthermore, 2,3-benzodiazepines can block responses mediated by kainate receptors, albeit with reduced potency compared with their effects on AMPA receptors,[21,27] but cyclothiazide and SCN^- are inactive at kainate receptors.[8,17,20,29] In our study there was a strong correlation between the magnitudes of the cyclothiazide and SCN^- effects, which is consonant with the concept that the two agents act at the same or related sites. This was supported by our results with recombinant AMPA receptor subunits where the GluR1 *flip* form was more sensitive to SCN^- block than the corresponding *flop* form. As noted, cyclothiazide exhibits a similar *flip* selectivity whereas 2,3-benzodiazepines do not.[9,19] However, a point mutation (S750Q) that eliminates cyclothiazide potentiation of $GluR1_{flip}$[17] has been shown to retain SCN^- sensitivity,[18] indicating that the determinants of cyclothiazide and SCN^- modulation, while likely similar, are not identical. Although the *flip/flop* module plays an important role in regulating the SCN^- sensitivity of AMPA receptors, other factors may also influence SCN^- responsiveness. Thus, we observed that coexpression of $GluR2_{flip}$ (which does not express as a homomer) with $GluR1_{flip}$ produced a dramatic increase in the sensitivity to SCN^- (Fig. 10). Similarly, Eugène *et al*.[8] found that the GluR2 subunit promoted the SCN^- sensitivity of recombinant *flop* subunits which by themselves were insensitive to the anion.

Do 2,3-benzodiazepines promote AMPA receptor desensitization?

Even if the 2,3-benzodiazepines and cyclothiazide act at distinct sites, it is still conceivable that the 2,3-benzodiazepines could exert their blocking action by promoting desensitization. In fact, in at least one study GYKI 52466 appeared to enhance AMPA receptor desensitization.[30] However, as we have shown previously[5] and confirmed in the present study, when perfusion techniques are used that are sufficiently rapid to resolve peak AMPA receptor currents, GYKI 52466 has no effect on the time-course of AMPA-evoked current responses, and thus does not appear to act by enhancing AMPA receptor desensitization. Similarly, GYKI 52466 had no effect on recovery from desensitization. In contrast SCN^- produced a dramatic speeding of AMPA responses and a slowing of recovery from desensitization, compatible with its action as a modulator of AMPA receptor desensitization.

CONCLUSION

The present studies have demonstrated that 2,3-benzodiazepines and SCN^-, two types of noncompetitive AMPA receptor antagonists, have distinct mechanisms and sites of action. SCN^- enhances AMPA receptor desensitization and may act at a site that overlaps the recognition site where cyclothiazide inhibits desensitization. Cyclothiazide can be considered to be an ''agonist'' at a ''cyclothiazide'' recognition site on the AMPA receptor that regulates desensitization. By analogy with the terminology commonly applied to the $GABA_A$ receptor, SCN^- would then be an ''inverse agonist'' at this site as it

produces a functional effect that is opposite to that of cyclothiazide. 2,3-Benzodiazepines, on the other hand, appear to act at a distinct recognition site via a mechanism unrelated to desensitization. These various distinct allosteric regulatory sites on AMPA receptors represent potentially important targets for therapeutic drugs.

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