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Characterization of YM90K, a selective and potent antagonist of AMPA receptors, in rat cortical mRNA-injected *Xenopus* oocytes

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Abstract

The inhibitory potencies of 6-(1*H*-imidazol-1-yl)-7-nitro-2,3(1*H*,4*H*)-quinoxalinedione hydrochloride (YM90K), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline (NBQX) and 1-(4-amino-phenyl)-4-methyl-7,8-methyl-endioxyl-5*H*-2,3-benzodiazepine (GYKI 52466) at excitatory amino acid receptors were examined in rat cortical mRNA-injected *Xenopus* oocytes using a two-electrode voltage clamp. Schild analysis of YM90K and NBQX inhibition of kainate currents yielded pA_2 values of 6.83 ± 0.01 and 7.24 ± 0.01, respectively. GYKI 52466 reduced the maximum kainate response and increased the kainate EC_{50} in a dose-dependent manner, suggesting that the antagonism of AMPA receptors by GYKI 52466 is mixed competitive and non-competitive for kainate. Schild analysis of YM90K and NBQX inhibition of kainate currents in the presence of 30 μM cyclothiazide yielded pA_2 values of 6.62 ± 0.03 (slope: 1.02 ± 0.01) and 7.10 ± 0.02 (slope: 1.00 ± 0.02), respectively, consistent with competitive antagonism. Cyclothiazide potentiated the AMPA response as well as the kainate response and increased the apparent Hill coefficients in a concentration-dependent manner. The potency of YM90K to inhibit AMPA-induced currents could be reduced by increasing the concentration of cyclothiazide. We showed that YM90K is a potent and competitive antagonist for AMPA receptors and the apparent affinity of competitive antagonists was reduced by cyclothiazide. Cyclothiazide can affect the interaction between receptors and both agonists and antagonists, suggesting that it might allosterically alter the affinity of agonists and competitive antagonists for their binding site on the AMPA receptor complex.

Keywords: Cyclothiazide; NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline); GYKI 52466; Allosteric regulation

1. Introduction

Research on the function of neuronal receptors for excitatory amino acids is becoming increasingly important to the understanding of normal and pathological brain function (Choi, 1988; Collingridge and Singer, 1990; McDonald and Johnston, 1990; Meldrum and Garthwaite, 1990). Three types of excitatory amino acid receptors associated with ion channels have been clearly distinguished, based largely on their sensitivity to agonists. The first, the NMDA/glycine receptor/channel, is activated by the combined action of two prototypical ligands, *N*-methyl-D-aspartate (NMDA) and glycine. The second, the AMPA receptor/channel, is activated by either (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate or domoate, while the third, the kainate receptor/channel, is activated by either kainate or domoate.

Characterization of AMPA receptor-mediated neurotoxicity has lagged behind our understanding of NMDA receptor-mediated excitotoxicity. Thus, the discovery in 1990 of the potent, selective and competitive AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)-quinoxaline (NBQX, Sheardown et al., 1990) was followed by extensive research into its effects in various animal models of glutamate pathology (Gill et al., 1992). Interest in these mechanisms has been further stimulated by the recent development of the AMPA receptor antagonists, 6-(1*H*-imidazol-1-yl)-7-nitro-2,3(1*H*,4*H*)-quinoxaline-dione hydrochloride (YM90K, Shimizu-Sasamata et al., 1996; Yatsugi et al., 1996), LY-293558 (Bullock et al., 1994) and 1-(4-amino-phenyl)-4-methyl-7,8-methyl-endioxyl-5*H*-2,3-benzodiazepine (GYKI 52466, Le Peillet et al., 1992; Smith and Meldrum, 1992). AMPA receptor agonists produce rapid desensitization of responses at the receptor (Trussel et al., 1988; Tang et al., 1989; Patneau

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and Mayer, 1991). This desensitization may safeguard against excessive glutamatergic transmission and thus limit the potential for neurotoxic damage (Zorumski et al., 1990). Long-term exposure of primary neuronal cultures to kainate causes marked neuronal cell death (Rothman et al., 1987; Koh et al., 1990; May and Robison, 1993). In contrast, similar treatment with AMPA results in negligible excitotoxicity (May and Robison, 1993; our unpublished data). With cyclothiazide, however, which is reported to block the desensitization of AMPA receptors (Yamada and Tang, 1993), treated cultures were vulnerable not only to kainate but also to AMPA (May and Robison, 1993; our unpublished data). Electrophysiologically, kainate can also produce desensitization but to a lesser degree than glutamate, and the steady state responses to kainate are 10 times larger than that to glutamate (Patneau et al., 1993). These results suggest that the magnitude of agonist-evoked steady state responses obtained in electrophysiological experiments on AMPA receptors correlates with the neurotoxic potential of the agonist used (Brorson et al., 1995). Although no endogenous analog of cyclothiazide in the brain has been identified, it is interesting to hypothesize that ischemia-induced neuronal death might result from impaired AMPA receptor desensitization resulting from the increased exposure to a cyclothiazide-like compound during and after ischemia. We therefore considered that clarification of the nature of interactions between AMPA receptors and their antagonists under conditions of normal and impaired receptor desensitization would help us better understand the mechanism of the neuroprotective actions of AMPA receptor antagonists. We now evaluated the inhibitory potency of the AMPA receptor antagonist, YM90K, at AMPA receptors expressed in *Xenopus* oocytes injected with rat cortical mRNA under conditions of normal and cyclothiazide-induced impaired receptor desensitization. YM90K was found to be a selective and potent AMPA receptor antagonist and was obtained by modification of the structure of the non-NMDA receptor antagonist, CNQX (Ohmori et al., 1994). The pharmacological properties of YM90K (Shimizu-Sasamata et al., 1996) and neuroprotective action in a cat middle cerebral artery-occlusion model (Yatsugi et al., 1996) are described elsewhere.

2. Material and methods

2.1. RNA extraction

Poly(A)$^+$ RNA was prepared from the cerebral cortex of male Wistar rats (6 weeks) by combined use of a total RNA Separator Kit and mRNA Separator Kit (Clontech Laboratories). mRNA was dissolved in water at a final concentration of RNA 1 mg/ml and stored as single-use aliquots at −80°C until injection into the oocytes.

2.2. Handling, injection, and culture of oocytes

Oocytes were obtained surgically from adult oocyte-positive female *Xenopus* frogs that were anesthetized by immersion in 0.3% Tricaine for 20 min and then placed on an ice bed. One or more lobes of ovary were removed into culture solution (Modified Barth's Saline, MBS) containing (in mM): 88 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 15 Hepes, 0.5 $CaNO_3$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, and supplemented with 10 mg/l each of penicillin and streptomycin and 40 mg/l gentamycin. The abdominal musculature, then the skin of the frog was sutured. The frog was allowed to heal for at least two weeks before more oocytes were removed. Oocytes were separated from the ovary manually with forceps and then injected with 25–50 ng of mRNA using a Drummond Nanoject. After injection, the oocytes were maintained in MBS at 19 °C. Four days after injection, they were treated with collagenase (type I, 2 mg/ml; Sigma) for 15 min at room temperature, and then the follicle cell layer was manually removed with fine forceps. The oocytes were then stored at 10°C, remaining viable for up to 11 days after their removal from the frog.

2.3. Electrophysiology

Experiments on oocytes were performed with a two-electrode voltage clamp (CA-1 high performance Oocyte clamp, Dagan, in earlier experiments; Axoclamp-2B, Axon Inst., in later experiments) at a holding potential of −60 mV in a continuously perfused chamber; flow rate was 5 ml/min. The extracellular solution contained modified Barth's solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.6), to which was added kainate or AMPA [(*RS*)-AMPA, Tocris Neuramin], cyclothiazide, or concanavalin A (Sigma). Electrodes with 1–5 MΩ resistance were filled with 3 M KCl. Current and voltage signals were recorded on a chart recorder and video tape recorder, and digitized with a COMPAQ computer for later analysis.

2.4. Data analysis

Agonist concentration-response curves were constructed by measuring the maximum current induced by increasing concentrations of agonist. Data from individual cells were fitted to the logistic equation:

$$I = I_{max} / \left\{1 + \left([EC_{50}]/\text{agonist}\right)^n\right\}$$

where I is the steady state current produced by [agonist]. The parameters I_{max} (maximum current) at infinite [agonist], n (the Hill coefficient), and EC_{50} (concentration of agonist producing 50% of I_{max}) were determined by a non-linear least squares curve-fitting program. The 95% confidence interval of the concentrations causing EC_{50} was

calculated as the logarithm of the drug concentration. Curves for the figures represent the average of 4–7 replicate experiments and in each cell 4–6 agonist concentrations were used to define the concentration-response relationship. Schild analyses were performed essentially as described (Verdoorn et al., 1989). Three concentrations of agonist were applied under control conditions, followed by three concentrations in the presence of a given concentration of antagonist. The ratio of equieffective agonist concentrations (dose ratio) was determined by interpolation of the linear regression of a fractional response versus the logarithm of the agonist concentration. The pA_2 and slope were determined for each cell by linear regression of the Schild plot and the mean values and standard errors of each separate determination are reported here. Differences between experimental groups were analyzed with Student's *t*-test.

YM90K, NBQX, GYKI 52466 and cyclothiazide were synthesized in our laboratories. All other reagents used were of analytical grade.

3. Results

3.1. Kainate current

We characterized the response to kainate in oocytes injected with native rat cortical mRNA. A full concentration-response curve for kainate was constructed as shown in Fig. 1A. The EC_{50} value (mean and 95% confidence interval) for kainate was 154.0 (134.3–176.6) μM and the apparent Hill coefficient was 1.44 ± 0.04 ($n = 6$). Kainate (1 mM)-evoked currents were reduced by co-application with AMPA (100 μM) (Fig. 1B). Moreover, the response to 10 μM kainate showed a 21.4-fold potentiation in the presence of 30 μM cyclothiazide, but no potentiation on pretreatment with 0.3 mg/ml concanavalin A for more than 3 min (Fig. 1C).

Cyclothiazide produced a leftward shift in the dose-response curve for kainate. Appropriate analysis revealed a decrease in the EC_{50} (mean and 95% confidence interval) for kainate from a control value of 154.0 (134.3–176.6) μM to 70.8 (61.2–81.8, six cells) μM and 42.1 (38.8–45.7, 14 cells) μM in the presence of cyclothiazide at 10 and 30 μM, respectively, with little change in the apparent Hill coefficient (control: 1.44 ± 0.04, cyclothiazide: 10 μM, 1.44 ± 0.04 and 30 μM, 1.44 ± 0.03). This left shift in the kainate concentration-response curve by cyclothiazide was also observed in cultured rat hippocampal neurons (Patneau et al., 1993) and in *Xenopus* oocytes expressing the Glu-A_{flip} receptor subunit (Partin et al., 1994). The EC_{50} (mean and 95% confidence interval)for potentiation of the response to 1 mM kainate by cyclothiazide was 4.9 (4.1–6.0, six cells) μM, with an apparent Hill coefficient of 1.15 ± 0.02. These pharmacological properties of the re-

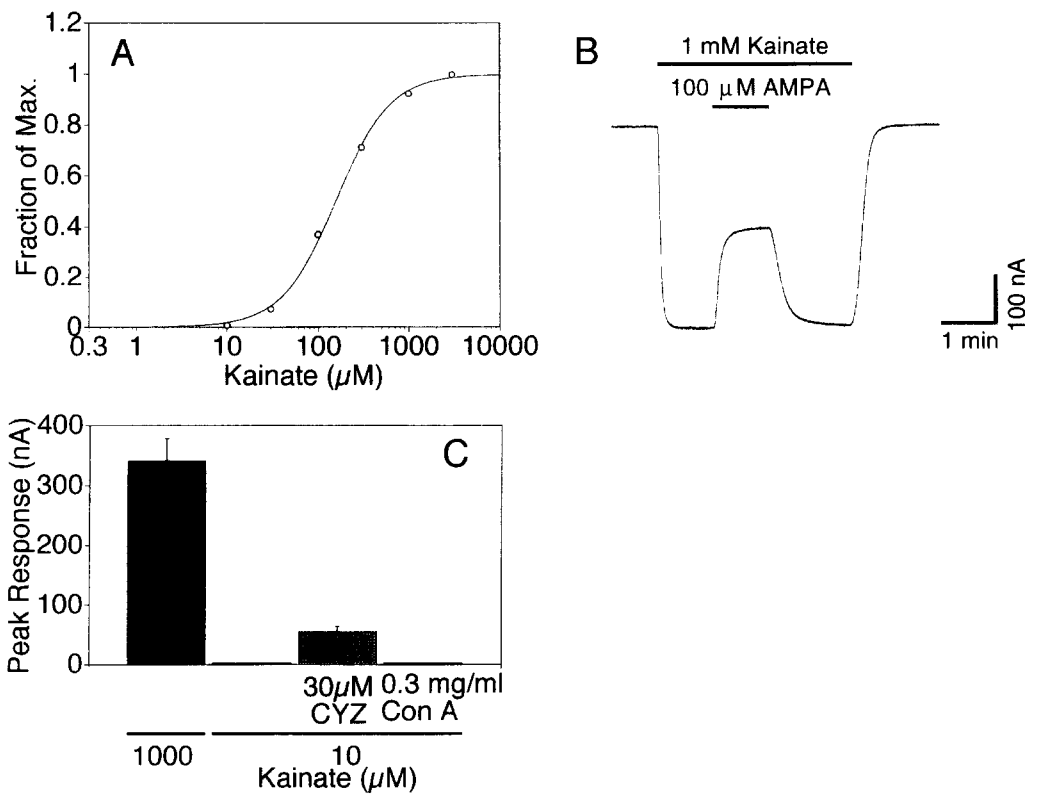

Fig. 1. Characterization of the current induced by kainate in mRNA-injected oocytes. (A) Concentration-response curve for kainate. The points represent the mean for 6 oocytes. (B) Inward current activated by 1 mM kainate recorded in the absence and presence of 300 μM AMPA. (C) The effects of cyclothiazide and concanavalin A (Con-A) on kainate-evoked currents. Cyclothiazide (CYZ) was applied for 1 min before and during 1 min application of 10 μM kainate and concanavalin A was applied for more than 3 min before kainate application. Data points show means ± S.E.M. of four observations.

sponses evoked by kainate in our assay system were shown to be similar to responses in neurons.

3.2. Antagonism of kainate-induced currents

Schild analysis was performed to determine the potency of YM90K and NBQX at AMPA receptors in oocytes. Fig. 2 shows a composite Schild plot obtained with data from six cells and three concentrations of YM90K and NBQX in the absence of cyclothiazide. Fig. 2B shows that the kainate concentration-response curves were shifted to the right by YM90K in a parallel manner. The Schild regressions of YM90K and NBQX were linear (Fig. 2C), with average slope values of 0.94 ± 0.01 and 0.91 ± 0.01, respectively, and mean pA_2 values of 6.83 ± 0.01 and 7.24 ± 0.01, respectively (Table 1).

Schild analysis confirmed the competitive character of the inhibition of cyclothiazide-potentiated kainate currents by YM90K and NBQX. These compounds induced parallel, rightward shifts of the concentration-response relations for kainate in the presence of cyclothiazide (Fig. 3A). The Schild regressions of YM90K and NBQX were linear (Fig. 3B), with an average slope of 1.02 ± 0.01 and 1.00 ± 0.02, respectively. The average pA_2 determined for YM90K and NBQX antagonism of cyclothiazide-potentiated kainate-induced currents were 6.62 ± 0.03 and 7.10 ± 0.02, respectively (Fig. 3B, Table 1). The potency of YM90K and NBQX to block kainate currents was less pronounced in the presence of cyclothiazide than in its absence.

Table 1
Potency of NBQX and YM90K at AMPA receptors in *Xenopus* oocytes injected with mRNA from rat cortex. This table summarizes the results of the Schild analyses using NBQX and YM90K. The pA_2 values (means ± S.E.M.) and slopes (means ± S.E.M.) shown here were derived from the means for individual cells, as described in Materials and methods

	Antagonist	pA_2	Slope
Kainate	NBQX	7.24 ± 0.01	0.91 ± 0.01 [a]
	YM90K	6.83 ± 0.01	0.94 ± 0.01 [a]
Kainate + 30 μM cyclothiazide	NBQX	7.10 ± 0.02 [b]	1.00 ± 0.02
	YM90K	6.62 ± 0.03 [b]	1.02 ± 0.01

[a] Significantly different from 1.0 ($P < 0.01$). [b] Significantly different from the same antagonist tested in the absence of cyclothiazide ($P < 0.01$).

The mechanism of blockade by GYKI 52466 was investigated by characterization of its effect on the concentration-response relationship for kainate. GYKI 52466 10 and 30 μM caused a concentration-dependent decrease in maximum current, with the kainate EC_{50} (mean and 95% confidence interval) increased from 154.0 (134.3–176.6) μM to 251.0 (203.0–310.2) μM by 10 μM GYKI 52466

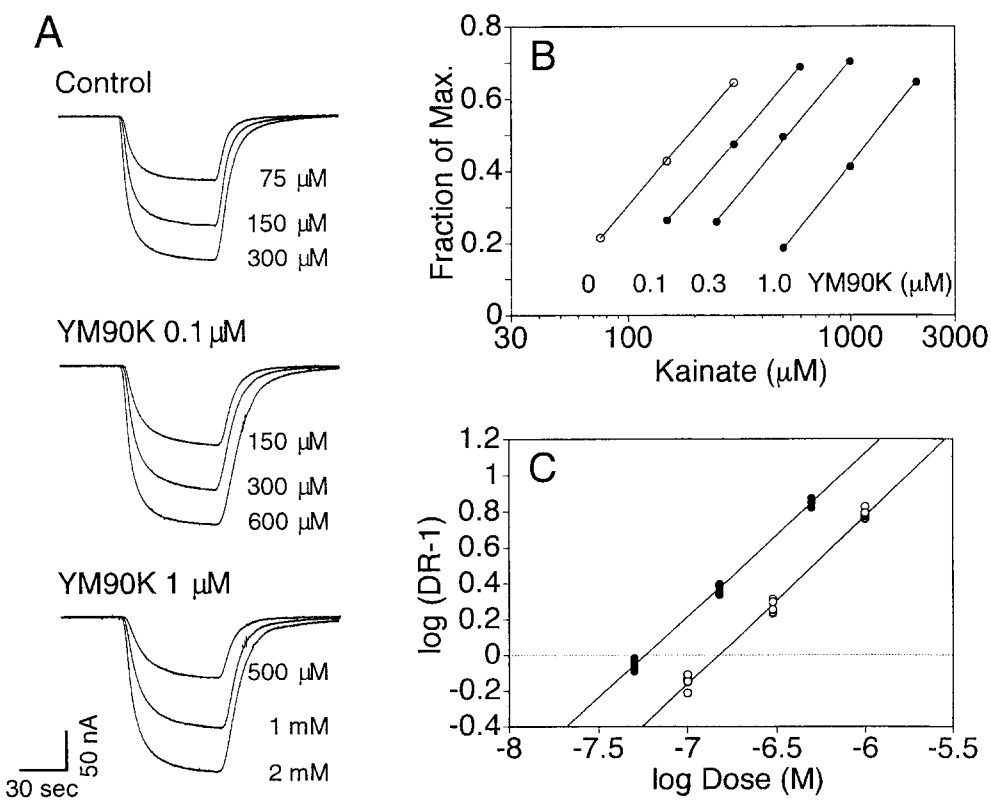

Fig. 2. Schild analysis of the antagonism by YM90K and NBQX of currents activated by kainate. (A) Inward currents activated by kainate and recorded from an oocyte. Control traces are shown at the *top*, followed by currents measured in the presence of YM90K. The shape of the responses was not qualitatively altered by YM90K. (B) Partial kainate concentration-response curves in the presence of increasing concentrations (0, 0.1, 0.3 and 1.0 μM) of YM90K. Each point represents the mean ± S.E.M. of six determinations. The error bars are smaller than the symbol sizes. The maximum response amplitude elicited by 3 mM kainate was tested before and after each curve was made. (C) Schild regressions of YM90K (○) and NBQX (●) antagonism of currents activated by kainate.

and to 351.4 (285.8–431.9) μM by 30 μM GYKI 52466. The antagonism of the AMPA receptor response to kainate by GYKI 52466 therefore appears to be mixed competitive and non-competitive. This non-parallel shift of concentration-response curves for kainate by GYKI 52466 is in close agreement with recent whole cell data for cultured superior colliculus neurons (Parsons et al., 1994). It remains to be seen whether GYKI 52466 affects the kainate recognition site by an indirect, allosteric interaction, although GYKI 52466 has no effect in the AMPA site ligand binding assays (our unpublished data).

3.3. Potentiation of response to AMPA by cyclothiazide

As shown in Fig. 4A, 0 ($n = 7$),10 ($n = 7$), 30 ($n = 5$) and 100 μM ($n = 4$) cyclothiazide caused a concentration-dependent increase in the maximal AMPA-induced current; moreover, apparent Hill coefficients (1.14 ± 0.02; 1.31 ± 0.04; 1.36 ± 0.03 and 1.43 ± 0.02, respectively) were also increased in a cyclothiazide concentration-dependent manner (Fig. 4B). The EC_{50} values of AMPA-induced currents were also changed by cyclothiazide [mean and 95% confidence interval: 26.7 (19.1–37.4) μM in the absence of cyclothiazide; 12.7 (10.6–15.2) μM with 10 μM cyclothiazide; 19.2 (14.3–25.7) μM with 30 μM cyclothiazide and 22.3 (16.4–28.3) μM with 100 μM cyclothiazide]. However, in contrast to kainate responses, the cyclothiazide concentration-dependence of the AMPA response was not clear. The EC_{50} value with 10 μM cyclothiazide was significantly lower than in its absence, but the EC_{50} values recovered as the concentration was increased.

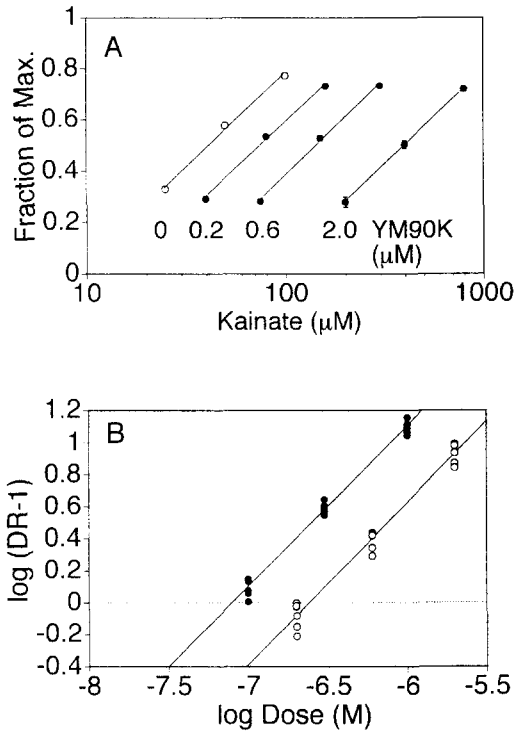

Fig. 3. Schild analysis of the antagonism by YM90K and NBQX of currents activated by kainate in the presence of cyclothiazide. (A) Partial kainate concentration-response curves in the presence of increasing concentrations (0, 0.2, 0.6 and 2.0 μM) of YM90K in the presence of 30 μM cyclothiazide. Each point represents the mean ± S.E.M. of six determinations. The error bars are smaller than the symbol sizes. The maximum response amplitude elicited by 3 mM kainate was tested before and after each curve was made. (B) Schild regressions of YM90K (○) and NBQX (●) antagonism of currents activated by kainate.

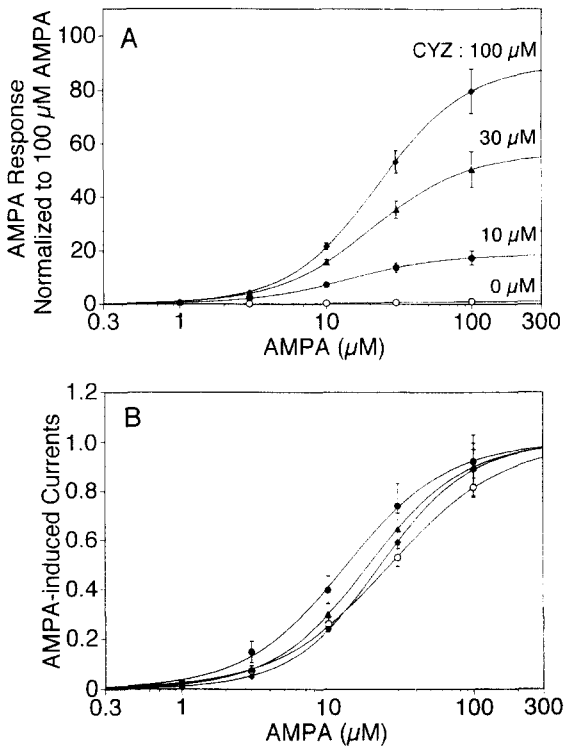

Fig. 4. Potentiation of response to AMPA by cyclothiazide. Concentration-response analysis for AMPA in the absence and presence of cyclothiazide (○: 0 μM, $n = 7$; ●: 10 μM, $n = 7$; ▲: 30 μM, $n = 5$; ◆: 100 μM, $n = 4$). (A) Data points show means ± S.E.M. of responses normalized to those evoked by 100 μM AMPA in the absence of cyclothiazide. (B) Each curve was first fitted with the equation given in the text. Maximal current, Imax, was then arbitrarily set at 1 and the extrapolated current amplitudes were expressed as relative values. The curves shown were obtained by reapplying the equation to the standardized average values.

3.4. Antagonism of AMPA-induced currents

We examined the effects of cyclothiazide on the potency of YM90K to block AMPA-induced currents (Fig. 5). AMPA was used at concentrations producing EC_{50} values in the presence of 10 or 100 μM cyclothiazide. The IC_{50} values estimated from inhibition curves were 142 ± 7 nM in the presence of 10 μM cyclothiazide and 353 ± 14 nM in the presence of 100 μM cyclothiazide. The potency of YM90K to block AMPA currents decreased as the concentration of cyclothiazide increased.

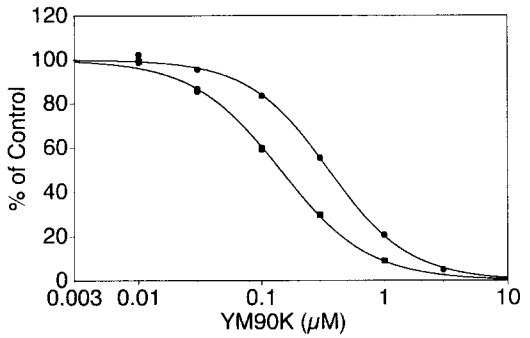

Fig. 5. Antagonism of AMPA responses by YM90K. Inhibition of AMPA-induced currents in the presence of cyclothiazide (■: 10 μM, $n = 5–14$; ●: 100 μM, $n = 5–13$) by YM90K. The curves were drawn using the equation: $I/I_{max} = 1/\{1 + ([antagonist]/IC_{50})^n\}$, with IC_{50} values of 142 μM for 10 μM cyclothiazide, 353 μM for 100 μM cyclothiazide.

Concentration-response curves for cyclothiazide and cyclothiazide with GYKI 52466 added are shown in Fig. 6. The data point for 300 μM cyclothiazide was excluded from the fit due to a decrease in potentiation at a high concentration of cyclothiazide, both in the presence and absence of GYKI 52466 (data not shown). Smooth curves were drawn from the fit of the data to the logistic equation (see Materials and methods). The EC_{50} (mean and 95% confidence interval) for the potentiation by cyclothiazide

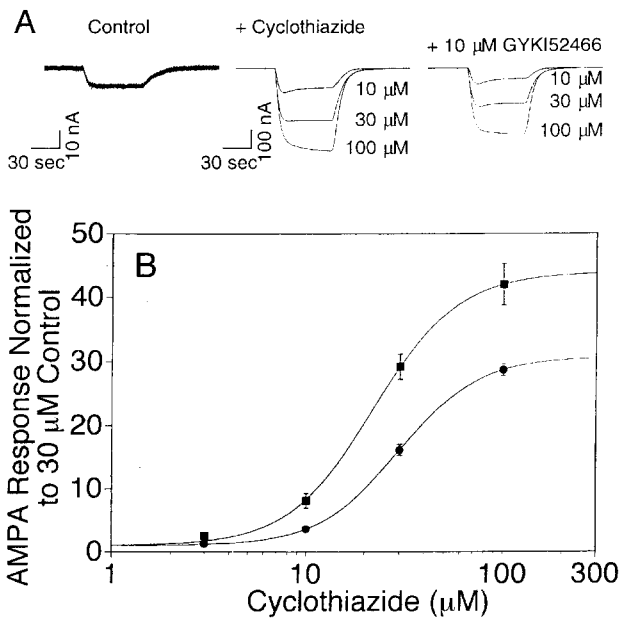

Fig. 6. Antagonism of AMPA responses by GYKI 52466. (A) Chart recordings of control AMPA-activated current (*left*), enhancement of AMPA-activated currents by cyclothiazide (*middle*) and inhibition of cyclothiazide-potentiated AMPA-activated current by GYKI 52466 (*right*). (B) Oocytes were exposed to increasing concentrations of cyclothiazide in the presence of fixed 30 μM AMPA, with (●) and without (■) 10 μM GYKI 52466. The points represent the means ± S.E.M. from six oocytes. The curves are the results of non-linear least squares fit of the data to the logistic equation and are plotted relative to 30 μM AMPA-induced current determined in the absence of GYKI 52466.

of responses to 30 μM AMPA was 21.5 (17.5–26.3) μM in control and 29.7 (26.3–33.6) μM in the presence of 10 μM GYKI 52466, with an apparent Hill coefficient of 2.09 ± 0.07 and 2.15 ± 0.08, respectively. The maximum possible potentiation calculated from the equation used to fit the concentration-response curves, assuming a saturating concentration of cyclothiazide, was 43.9-fold in control and 30.7-fold in the presence of 10 μM GYKI 52466. The maximum response was reduced by 30%.

4. Discussion

It has been shown that *Xenopus* oocytes are a useful model for the investigation of the pharmacological effects of drugs on neuronal channels via the measurement of whole-cell currents (Parker et al., 1986). The pharmacological and physiological properties of the responses evoked by excitatory amino acid receptor agonists in oocytes injected with rat brain mRNA were shown to be similar to those seen in neurons (Verdoorn and Dingledine, 1988). We characterized the responses to kainate in oocytes injected with native rat cortical mRNA. The currents induced by kainate were potentiated by cyclothiazide but not concanavalin A, and were reduced by AMPA. These characteristics are nearly identical to those found for Glu_{1-4} receptor subunits (Keinänen et al., 1990; Partin et al., 1993), suggesting that the inward current evoked by kainate was caused by the activation of AMPA receptors.

We evaluated the potency of the AMPA receptor antagonists, YM90K and NBQX, in the inhibition of kainate-evoked currents in *Xenopus* oocytes injected with rat cortical mRNA under conditions of normal and cyclothiazide-induced impaired receptor desensitization. The rank order of antagonist potency (NBQX > YM90K) both in the absence and presence of cyclothiazide reflects the order of K_i values for inhibition by YM90K and NBQX of [^{3}H]AMPA binding to rat cortical membrane preparations, 0.084 and 0.060 μM, respectively (Shimizu-Sasamata et al., 1996). The parallel shift in the log concentration-response curve to kainate and the near unity values of the Schild plots are consistent with the suggestion that YM90K, like NBQX, acts as a competitive antagonist at kainate recognition sites. We did find, however, that the slope of the Schild plot was shallow in the absence of cyclothiazide for both of these antagonists. Shallow Schild plot slopes have been reported for other competitive AMPA receptor antagonists (Fletcher et al., 1988; Blake et al., 1989; Desai et al., 1995), but the cause of this phenomenon is not known.

We found that cyclothiazide decreased the apparent affinity of competitive antagonists to inhibit kainate- and AMPA-induced currents. A recent study showed that cyclothiazide reduces NBQX blockade of AMPA receptor-mediated excitatory postsynaptic currents and AMPA-in-

duced currents (Rammes et al., 1995, 1996). These results suggest that cyclothiazide allosterically modulates competitive AMPA antagonist binding to its recognition site. An allosteric interaction between two distinct sites on ionotropic glutamate receptor complexes has also been described for the NMDA receptor. Agonist compounds acting at the strychnine-insensitive glycine site on the NMDA receptor are known to alter the affinity of other compounds acting at the glutamate recognition site (Monaghan et al., 1988; Fadda et al., 1988; Kaplita and Ferkany, 1990). The effect of cyclothiazide on the affinity of competitive antagonists for recognition sites should be tested directly when a radioactive ligand of a competitive antagonist on the AMPA receptor becomes available.

The antagonism of AMPA receptors by GYKI 52466 appears to be non-competitive for cyclothiazide, suggesting that the binding sites for GYKI 52466 on AMPA receptors might not be the same as those for cyclothiazide. Previous studies have suggested that cyclothiazide may interact at the 2,3-benzodiazepine site on the AMPA receptor complex (Palmer and Lodge, 1993; Zorumski et al., 1993). In these reports, cyclothiazide competitively reversed the inhibition by GYKI compounds in cortical slices (Palmer and Lodge, 1993) and hippocampal neurons (Zorumski et al., 1993). This point remains controversial (Rock and Campbell, 1993; Rammes et al., 1994, 1995, 1996) and it is difficult to be certain whether the 2,3-benzodiazepaine recognition site is the same as the cyclothiazide binding site. Cyclothiazide at high concentrations has a neurotoxic effect on rat hippocampal neurons even in the presence of competitive AMPA antagonists, indicating that cyclothiazide has unknown cytotoxic actions, not exerted through AMPA receptors (our unpublished data). We were therefore only able to make partial concentration dependence curves for the potentiation of AMPA-induced currents. Much additional work will be needed to fully elucidate the mechanism of action of 2,3-benzodiazepines.

The present study showed that cyclothiazide allosterically modulates kainate and AMPA binding to their recognition sites on AMPA receptors. While the apparent affinity for kainate was increased by cyclothiazide in a concentration-dependent manner, the effect of cyclothiazide on AMPA binding sites appeared to be more complex than the effect on kainate binding sites. While cyclothiazide at a low concentration increased the apparent affinity for AMPA, higher concentrations caused a decrease in apparent affinity for AMPA and an increase in Hill coefficient. Unfortunately, under conditions used in physiological experiments (absence of thiocyanate), [^{3}H]AMPA binding is very slight and the effect of cyclothiazide on AMPA binding sites has not been clarified. Our present results, however, suggest the existence of more than one mechanism by which cyclothiazide modulates the interaction between AMPA and its recognition sites on AMPA receptors, although the mechanism of action of cyclothiazide at AMPA binding sites is not clear.

Cyclothiazide has been used as a powerful new tool in the analysis of the allosteric regulatory mechanisms at AMPA receptors (Patneau et al., 1993; Rammes et al., 1995, 1996). We confirmed that cyclothiazide caused a left shift in the kainate concentration-response curve and found that it increased the apparent Hill coefficient of AMPA responses and decreased the potency of antagonists in the inhibition of agonist-induced currents in rat cortical mRNA-injected *Xenopus* oocytes. The results of our experiments provide further evidence for the existence of allosteric interactions between cyclothiazide and both agonist and competitive antagonist recognition sites on the AMPA receptor complex.

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