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Differential Antagonism of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid-Preferring and Kainate-Preferring Receptors by 2,3-Benzodiazepines

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SUMMARY

Whole-cell recordings were used to study the antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-preferring and kainate-preferring receptors by 2,3-benzodiazepines. Current through kainate-preferring receptors was recorded in rat dorsal root ganglion (DRG) neurons,whereas AMPA receptor current was measured in cultured neurons from rat cerebral cortex. In both cell types 2,3-benzodiazepines produced noncompetitive inhibition; however, antagonist potency was much higher against AMPA-preferring receptors than against kainate receptors. The most potent compound, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine (GYKI 53655), blocked AMPA receptor currents with an IC_{50} of approximately 1 μM. A second benzodiazepine, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466), was about 20-fold less potent at AMPA receptors (IC_{50} = 18 μM). Both drugs were markedly weaker against kainate currents in DRG neurons. At 200 μM, the highest concentration tested, GYKI 53655 and GYKI 52466 produced only 30–40% inhibition in DRG cells, suggesting that for both compounds the IC_{50} against kainate receptors is >200 μM. Our study suggests that GYKI 53655, at a concentration of approximately 10 μM, should produce >90% block of AMPA-preferring receptors but <5% inhibition of kainate-preferring receptors. Because the antagonism by this drug is noncompetitive, its effectiveness should not be influenced by phasic changes in transmitter concentration, making it an ideal compound for functional studies of the role of kainate and AMPA receptors in synaptic transmission.

Many cells in the nervous system express several different receptors for glutamate, including at least three different subtypes that form ionic channels, i.e., the NMDA receptor and two classes of non-NMDA receptors, one that has high affinity for the agonist AMPA and the other that prefers the agonist kainate (1–4). Both the NMDA receptor and the AMPA receptor are known to serve as postsynaptic receptors at fast excitatory synapses (2, 3). Although the function of kainate receptors is not yet established, one form of this receptor is expressed by a subset of peripheral sensory neurons in rat DRGs (5, 6). The DRG cells do not express either the NMDA- or AMPA-preferring receptors, making this a good preparation to study kainate receptors in isolation (6–8).

In the past few years the pace of work on glutamate receptors has accelerated, spurred by the cloning of a large number of subunits of these receptors (9) and by the growing realization that hyperactivation of glutamate receptors may be involved in neuronal pathology (10). Analysis of currents produced when cloned receptor subunits are expressed in *Xenopus* oocytes or in mammalian cell lines suggests that the subunits designated GluR1–4 contribute to AMPA-preferring receptors, whereas kainate-preferring receptors appear to be derived from subunits GluR5–7, possibly in combination with subunits KA1 and KA2 (9). Several lines of evidence indicate that native receptors are heteromeric assemblies composed of several different subunits (9, 11, 12), but the exact molecular composition of native channels has not been firmly established for any cell type.

Pharmacological studies of native AMPA and kainate receptors have demonstrated that both receptor subtypes can be activated by the same collection of agonists, including AMPA, kainate, glutamate, quisqualate, domoate, and a

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; DRG, dorsal root ganglion; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GYKI 53655, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine HCl; GYKI 52895, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine; EBSS, Earle's balanced salt solution; Con A, concanavalin A; CNS, central nervous system; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GluR1–7, glutamate receptor types 1–7; KA1 and KA2, kainate receptor types 1 and 2.

number of willardine derivatives (6, 13), but the relative potencies of these compounds are markedly different at the two receptor subtypes. A second clear difference is seen in the susceptibility of the receptors to modulation by benzothiadiazides and related compounds. AMPA-preferring receptors are strongly potentiated by these drugs, whereas kainate-preferring receptors display modest inhibition at high doses (e.g., >50 μM cyclothiazide) (7, 14–16). Most of the antagonists that act at non-NMDA receptors show relatively poor selectivity between AMPA and kainate receptors (4), although a few compounds clearly exhibit some degree of differential potency (17–21). We undertook the present study to examine the selectivity of a series of 2,3-benzodiazepines that have recently been shown to inhibit non-NMDA receptors expressed by hippocampal neurons (22–25). Our results demonstrate that GYKI 53655 blocks currents elicited at AMPA-preferring receptors on cortical neurons with at least 200-fold greater potency, compared with those at kainate-preferring receptors on DRG cells.

Materials and Methods

Cell preparation. DRGs were dissected from the lumbar and thoracic regions of newborn to 3-week-old Long Evans rats and were collected in EBSS (no. 14160; GIBCO-BRL) containing 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, and 26 mM $NaHCO_3$ (equilibrated with 5% CO_2/95% O_2). The ganglia were trimmed and placed in EBSS with 1 mg/ml protease type XXIII (Sigma). After gentle stirring for 20 min at 30–35° under a stream of 5% CO_2/95% O_2, the ganglia were rinsed two or three times in EBSS containing 1 mg/ml bovine serum albumin (A-7030; Sigma Chemical Co.) and 1 mg/ml trypsin inhibitor (T-9253; Sigma) and were triturated with a fire-polished Pasteur pipette. Periodically the ganglia were allowed to settle to the bottom of the tube and dissociated neurons, which remained suspended in solution, were transferred to a second tube. The dissociated cells were kept overnight in this second tube at room temperature and used the following day. Cortical cells were isolated from newborn Long-Evans rats and maintained in culture as described previously (26). Recordings were obtained from cortical neurons after 6–14 days in culture.

Electrical recording and drug application. Kainate-induced currents were recorded using the whole-cell configuration of the patch-clamp technique. Pipette resistance ranged from 1 to 10 MΩ with an internal solution containing 10 mM HEPES, 10 mM EGTA, 5 mM CsCl, and 140 mM $CsCH_3SO_3$ or CsF, titrated to pH 7.40 with CsOH. Currents were recorded with an Axopatch 200 amplifier (Axon Instruments) and filtered at 1–5 kHz. For storage and analysis, the data were compressed by averaging 3 msec of current at 0.1–1-sec intervals. For bulk perfusion of the recording chamber, we used Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.40). The external solution for drug application contained 160 mM NaCl, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.40, 500 nM tetrodotoxin, and 2 μM dizocilpine. Control, agonist, and antagonist solutions were applied from a bank of microcapillary tubes mounted on a micromanipulator and connected to a series of reservoirs. Solution flow was driven by gravity. To block desensitization of kainate currents in DRG cells (6), Con A was applied at 2 μM for 5–10 min before recording.

Antagonists were prepared as 10–40 mM stock solutions in dimethylsulfoxide or, in some cases (dizocilpine), ethanol. These stock solutions were diluted into control or agonist-containing solutions so that the final concentration of vehicle was ≤0.5%. Pure dimethylsulfoxide or ethanol was added to solutions lacking antagonist, so that all of the solutions used in each experiment contained the same levels of vehicle. All drug stock solutions were stored at −20°. AMPA, GYKI 52466, and GYKI 52895 were purchased from Research Biochemicals. Kainate, tetrodotoxin, and Con A were purchased from Sigma. Cyclothiazide and GYKI 53655 were kindly provided by Eli Lilly and Co.

Results

Whole-cell currents elicited by kainate were used to evaluate the inhibitory potency of three different 2,3-benzodiazepines, i.e., GYKI 53655, GYKI 52466, and GYKI 52895 (22, 27). Previous work (e.g., Refs. 28 and 29) has shown that, in hippocampal cells, cortical neurons, and many other CNS cell types, kainate activates AMPA-preferring receptors to produce a large maintained current. In contrast, kainate-preferring receptors expressed by DRG neurons undergo strong desensitization to excitatory amino acids (6, 7, 13). Brief exposure of DRG cells to the lectin Con A results in virtually complete suppression of desensitization, without any apparent change in the agonist concentration required to yield half-maximal receptor activation (6, 13). Because nondesensitizing currents are much more suitable for quantitative pharmacological analysis, most of our experiments were carried out with DRG neurons that had been treated with Con A. Nevertheless, all of the observations were verified qualitatively in freshly isolated cells that had not been exposed to lectin.

Fig. 1 illustrates the inhibition of kainate current in DRG and cortical neurons by GYKI 52466 and GYKI 53655. Application of the GYKI compounds alone had no effect on the steady holding current (see Fig. 3); however, addition of the drugs to solutions containing kainate resulted in clear inhibition of currents recorded in cortical cells (Fig. 1, A and B) and DRG neurons (Fig. 1, C and D). As previously described for hippocampal neurons (23, 25), the onset and recovery from block were relatively rapid in both DRG and cortical cells. Concentration-inhibition curves for the three antagonists are shown in Fig. 2A. In cortical neurons, GYKI 52466 inhibited AMPA-preferring receptors with an IC_{50} of approximately 20 μM. The 3-*N*-methylcarbamyl derivative GYKI 53655 displayed >20-fold higher potency, whereas the 3,4-dihydro derivative GYKI 52895 was considerably less potent (22, 25) (Table 1).

In contrast to the potent inhibition observed in cortical cells, GYKI 53655 and GYKI 52466 were much less effective against kainate currents recorded in DRG neurons. Both drugs produced <50% inhibition at concentrations up to 200 μM, which is close to the solubility limit for these compounds in physiological solutions. The inhibition produced by the GYKI compounds in both DRG and cortical neurons was independent of the concentration of kainate used to elicit current. Table 2 presents the antagonism obtained for several antagonist concentrations when either 100 μM or 1 mM kainate was used to evoke the current. There was no significant difference in block between the two agonist concentrations, except for inhibition by GYKI 52466 in DRG cells, which showed a slight reduction at 1 mM kainate. Fig. 2B shows the full concentration-response relations for kainate in DRG neurons in the presence and absence of 200 μM GYKI 52466 or GYKI 53655. The smooth curves are the best fits of a noncompetitive model of inhibition. Departure from the model was not significant for either drug. For comparison, the data for 100 μM and 1 mM kainate from Table 2 are plotted in Fig. 2B. These points illustrate the fact that there

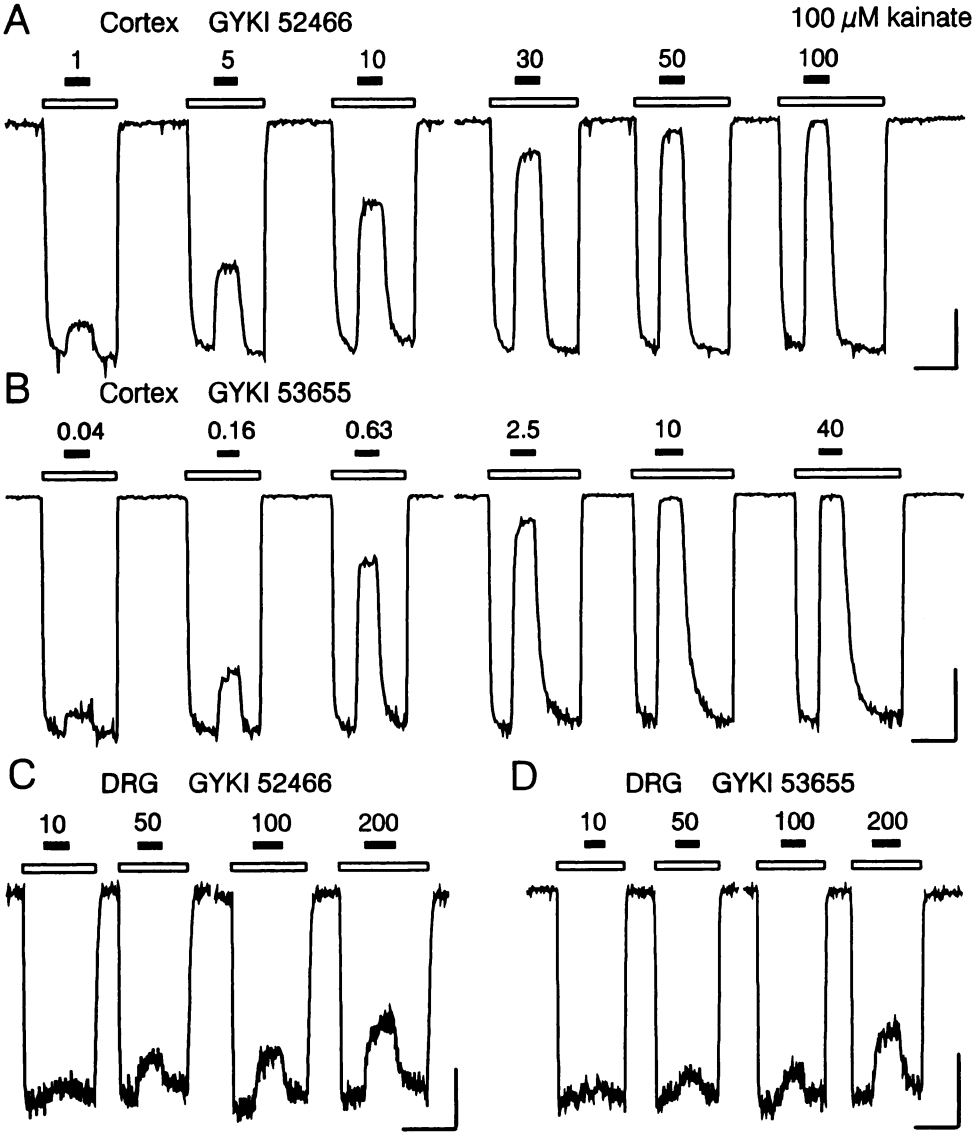

Fig. 1. Inhibition of kainate current in DRG and cortical neurons by GYKI 52466 and GYKI 53655. *Open bars*, kainate (100 μM) application; *filled bars*, coapplication of GYKI 52466 (A and C) or GYKI 53655 (B and D). Antagonist concentration (in μM) is shown above each bar. Holding potential, −60 mV. A and B, Cortical neurons; C and D, DRG neurons. *Scales*, A, B, and D, 100 pA and 30 sec; C, 50 pA and 30 sec.

was some degree of variability in the percentage block from day to day.

As shown in Fig. 3, the level of steady state blockade at +40 mV was similar to that obtained with holding at −60 mV. Table 3 contains data for DRG neurons and cortical cells that were tested with drug applications at both −60 mV and +40 mV. There was no significant difference in the levels of inhibition at these two potentials. In addition, there was no evidence for a use-dependent component of inhibition when cells were equilibrated with GYKI compounds before the application of kainate (data not shown).

Fig. 4 shows the effect of 30–200 μM cyclothiazide on kainate current in DRG cells treated with Con A. In agreement with the work of Wong and Mayer (7), who studied desensitizing kainate currents in freshly isolated cells, we found that cyclothiazide produced only modest inhibition of kainate-preferring receptors, with no evidence for the dramatic potentiation that is seen at AMPA receptors expressed by CNS neurons (7, 16, 30). This weak inhibition produced by cyclothiazide was not tested for possible dependence on agonist concentration or membrane voltage.

Discussion

Selective antagonism. 2,3-Benzodiazepines have attracted increasing interest since the initial report of their ability to inhibit spinal reflexes (27). Two recent studies (23, 24) have demonstrated that these compounds produce noncompetitive inhibition of non-NMDA receptor channels expressed by cultured hippocampal neurons. In addition, it has been suggested (24, 31, 32) that 2,3-benzodiazepines may inhibit the action of compounds such as cyclothiazide (7, 30) and aniracetam (14), which potentiate AMPA receptor currents by reducing desensitization. Because the potentiating actions of cyclothiazide and aniracetam are known to be restricted to AMPA-preferring receptors (7, 16), we chose to examine whether 2,3-benzodiazepines might exhibit selectivity between AMPA and kainate receptor subtypes (see also Ref. 34). The major finding of our study is that GYKI 53655 displays >200-fold higher potency against AMPA-preferring receptors found on cultured rat cortical neurons than against the kainate-preferring subtype expressed by freshly dissociated rat DRG neurons.

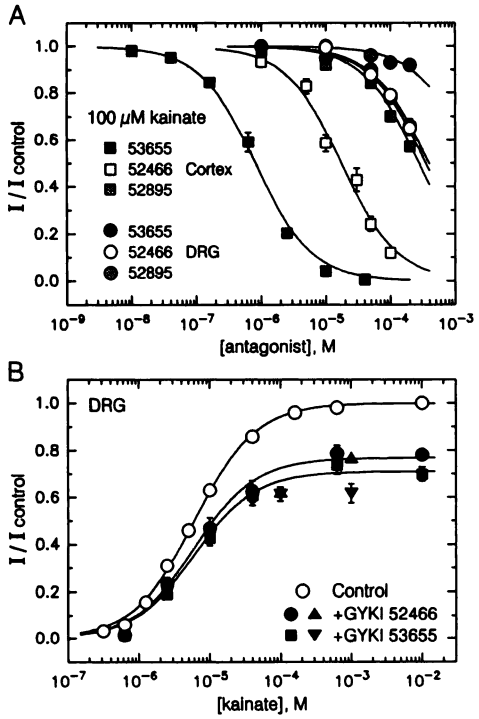

Fig. 2. Inhibition of kainate current in DRG and cortical neurons by GYKI compounds. A, Currents activated by 100 μM kainate in the presence of each inhibitor are plotted as a fraction of the control current evoked by 100 μM kainate alone. *Smooth curves*, best fits of $I/I_{control} = 1/(1 + [antagonist]/IC_{50})$. IC_{50} values were as follows: cortex, 0.82 μM (GYKI 53655), 18 μM (GYKI 52466), and 250 μM (GYKI 52895); DRG, 374 μM (GYKI 53655), 378 μM (GYKI 52466), and 2 mM (GYKI 52895). Except for the results with GYKI 53655 and GYKI 52466 in cortical neurons, these values should be considered estimates, due to uncertainty associated with fitting partial concentration-inhibition data. B, Currents activated by increasing concentrations of kainate alone (○) or in the presence of 200 μM GYKI 52466 (●) or GYKI 53655 (■). *Smooth curves*, best simultaneous fits of the equation $I/I_{control} = (1 - f_{drug})/[1 + (EC_{50}/[agonist])^n]$ to all three concentration-response relations. This equation has four free parameters, as follows: half-maximal agonist concentration, EC_{50} = 6 μM (95% confidence interval, 5–7 μM); slope factor, n = 1.0 (0.9–1.1); fractional block by GYKI 52466, f_{52466} = 0.23 (0.20–0.26); and fractional block by GYKI 53655, f_{53655} = 0.29 (0.26–0.32). Separate fits of the logistic equation to each curve were not significantly better, as determined by the ratio of residual variance ($F_{4,141}$ = 1.025). For comparison, data points from Table 2 for 100 μM and 1 mM kainate, which were collected using cells and solutions prepared on different days, are shown (▲, ▼). These points were not used to fit the parameters listed above.

Noncompetitive inhibition. Our IC_{50} of 18 μM (GYKI 52466) is in fairly close agreement with that obtained in cultured hippocampal neurons (23, 24). The higher potency of the 3-*N*-methylcarbamyl derivative was reported by Tarnawa *et al.* (22) and is consistent with the potent inhibition observed by Palmer and Lodge (32) and Donevan *et al.* (25). We saw little evidence for voltage dependence in the antagonism produced by either drug (23) or for relief of inhibition by a 10-fold increase in agonist concentration (23, 24). Thus, it seems unlikely that these compounds directly interfere with agonist binding or act by plugging the conduction pathway. A number of laboratories have obtained evidence that the inhibition of AMPA-preferring receptors by GYKI 52466 or GYKI 53655 can be overcome by addition of cyclothiazide (24, 32, 35) or aniracetam (31), suggesting that all of these compounds might act at a common modulatory site that is allosterically coupled to channel gating. This point remains controversial (35, 36), however, and much additional work will be needed to fully elucidate the mechanism of action of benzodiazepines as well as cyclothiazide and aniracetam. The recent demonstration (33) that AMPA receptor flip/flop splice variants display differential sensitivity to modulation by cyclothiazide indicates yet another level of complexity. In future work, it will be important to determine whether alternate splicing has any effect on inhibition by GYKI compounds or on their ability to interfere with benzothiadiazides and other modulators.

At this point it is difficult to be certain whether the mechanism of inhibition by the benzodiazepine antagonists is the same at both AMPA-preferring and kainate-preferring receptors. Because of solubility limitations, we were only able to construct partial concentration-inhibition curves for the currents recorded in DRG neurons. Nevertheless, the inhibition we observed in DRG cells was similar to that seen in CNS neurons, in that it was independent of membrane potential as well as agonist concentration. As previously reported by Wong and Mayer (7), we found that in DRG cells cyclothiazide did not enhance kainate currents but instead produced mild inhibition at concentrations of >50 μM. Taken together,

TABLE 1

GYKI compounds inhibit AMPA and kainate receptors

IC_{50} values were obtained from the best fit of the equation $I/I_{control} = 1/(1 + [antagonist]/IC_{50})$ to concentration-inhibition data, as shown in Fig. 2. Conservative estimates are given when only partial curves could be obtained due to solubility limitations.

Antagonist	IC_{50}	
	Cortex	DRG
	μM	
GYKI 53655	0.8[a]	>200
GYKI 52466	18[b]	>200
GYKI 52895	>200	>1000

[a] 95% confidence interval, 0.70–0.95 μM.
[b] 95% confidence interval, 15–20 μM.

TABLE 2

Inhibition by GYKI compounds is independent of agonist concentration

Steady state inhibition was determined with holding at −60 mV. Values are mean ± standard error. The number of cells tested is given in parentheses. Differences between the means were not significant except as indicated.

Antagonist	Inhibition versus 100 μM kainate	Inhibition versus 1 mM kainate
	%	%
GYKI 53655		
Cortex		
160 nM	14 ± 3 (n=8)	16 ± 3 (n= 7)
630 nM	37 ± 3 (n=9)	36 ± 2 (n= 9)
2.5 μM	78 ± 3 (n=6)	75 ± 4 (n= 9)
DRG		
100 μM	21 ± 2 (n=8)	25 ± 2 (n= 3)
200 μM	35 ± 3 (n=8)	38 ± 4 (n= 3)
GYKI 52466		
Cortex		
5 μM	14 ± 2 (n=7)	20 ± 3 (n= 9)
30 μM	53 ± 2 (n=6)	47 ± 5 (n= 7)
100 μM	86 ± 1 (n=5)	90 ± 2 (n= 7)
DRG		
100 μM	22 ± 2 (n=8)	14 ± 1 (n=7)[a]
200 μM	34 ± 3 (n=8)	23 ± 1 (n=7)[a]

[a] Difference is significant at $p < 0.05$ (two-tailed t test).

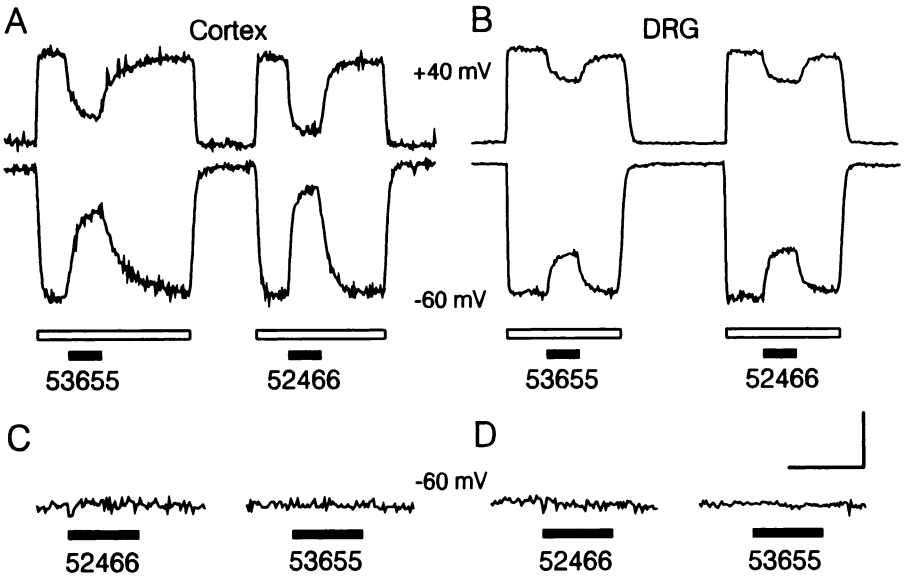

Fig. 3. Inhibition of kainate current by GYKI compounds at +40 and −60 mV. A and B, *Open bars*, kainate (100 μM) application; *filled bars*, coapplication of GYKI 52466 (A, 40 μM; B, 200 μM) or GYKI 53655 (A, 2 μM; B, 200 μM). C and D, *filled bars*, application of antagonists alone, at −60 mV (52466: C, 50 μM; D, 200 μM; 53655: C, 2.5 μM; D, 200 μM). A and C, Cortical neurons; B and D, DRG neurons. *Scales*, A, 150 pA and 45 sec (*top trace*) or 25 sec (*bottom trace*); B, 200 pA and 20 sec; C and D, 50 pA and 5 sec.

TABLE 3

Inhibition by GYKI compounds is independent of holding potential

Steady state inhibition of current gated by 100 μM kainate was determined with holding at −60 mV or +40 mV. The antagonist concentration is given in parentheses. Values are mean ± standard error. Differences between the means were not significant at $p < 0.05$ (two-tailed *t* test).

Antagonist	Inhibition at −60 mV	Inhibition at +40 mV	*n*[a]
	%	%	
GYKI 53655			
Cortex (2 μM)	69 ± 2	71 ± 1	7
DRG (200 μM)	31 ± 2	27 ± 2	5
GYKI 52466			
Cortex (40 μM)	82 ± 2	79 ± 1	7
DRG (200 μM)	30 ± 2	28 ± 2	6

[a] *n*, number of cells tested at both potentials.

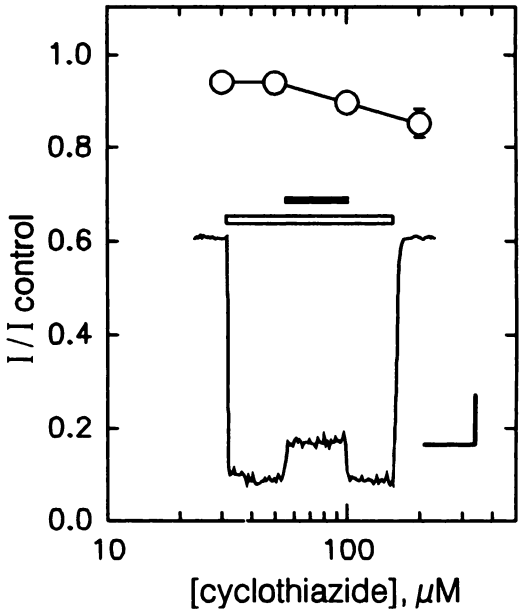

Fig. 4. Inhibition of kainate current in DRG neurons by cyclothiazide. Current activated by 100 μM kainate in the presence of cyclothiazide is plotted as a fraction of control current versus cyclothiazide concentration. All cells were treated with Con A ($n = 7$). *Inset*, sample trace showing inhibition of kainate current by 200 μM cyclothiazide. *Open bar*, kainate (100 μM) application; *filled bar*, simultaneous application of cyclothiazide. Holding potential, −60 mV. *Scale*, 100 pA and 10 sec.

these results suggest the provisional conclusion that both kainate-preferring and AMPA-preferring receptors possess one or more binding sites that accept benzodiazepines and benzothiadiazides. At kainate receptors expressed by DRG cells, the apparent affinities for both types of molecules are substantially lower than at AMPA receptors and cyclothiazide inhibits channel function rather than causing potentiation. As discussed below, DRG neurons that are sensitive to excitatory amino acids appear to express the GluR5 and KA2 subunits. Further work will be needed to determine whether the same pattern of inhibition is found for kainate receptors resulting from other subunit combinations, which are likely to be expressed by other cell types in the nervous system.

Implications for functional studies. Early indications that kainate and AMPA (originally quisqualate) act on two different receptor populations (1) have subsequently been borne out by the molecular cloning of distinct subfamilies of non-NMDA receptor subunits (9). It is now well established that AMPA-preferring receptors mediate transmission at fast excitatory synapses throughout the CNS (2), but the functional role of kainate receptors remains one of the unsolved mysteries of excitatory amino acid research (37). Efforts to determine the function that kainate receptors serve in the operation of the nervous system have been hampered by the lack of highly selective agonists and antagonists; the overlap between AMPA- and kainate-preferring receptors that is observed for most agonists and antagonists makes it very difficult to draw firm conclusions from pharmacological experiments *in situ*. In addition, it has proven quite challenging to demonstrate kainate receptor currents in CNS cell populations that appear to express kainate receptor subunits based on *in situ* hybridization or immunocytochemical localization (21, 37).

Primary afferent C fibers, which arise from small-diameter sensory neurons, exhibit a pure population of kainate-preferring receptors (5, 8). Molecular studies (12, 16, 38, 39) suggest that GluR5 and KA2 are the subunits most likely to make up these receptors. It has been proposed (5) that the

kainate receptors expressed by DRG neurons reside on their presynaptic axon terminals in the spinal cord and serve to influence primary afferent transmission. Direct experimental evidence for a functional role of these receptors in afferent transmission is lacking, however, and it remains possible that the kainate receptors may serve some function in the periphery (40).

The results of the present study suggest that GYKI 53655 may be a promising drug for functional studies *in situ*. Although this compound inhibits kainate receptors at high concentrations, the potency against AMPA-preferring receptors is >200-fold greater, such that a concentration of approximately 10 μM GYKI 53655 should produce >90% inhibition of AMPA receptors, while causing <5% blockade of kainate receptors. In addition, the noncompetitive mechanism of inhibition means that changes in synaptic glutamate concentration would not affect the level of blockade, as would be the case for a competitive antagonist.

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