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Allosteric interactions between cyclothiazide and AMPA/kainate receptor antagonists

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1 Cyclothiazide blocks α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor desensitization and potentiates AMPA receptor gated currents. Interactions between cyclothiazide, and the non-competitive antagonist GYKI52466 (GYKI) and competitive antagonist 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo (F) quinoxaline (NBQX) were studied at native and recombinant AMPA/kainate receptors using whole-cell recording in order to characterize the modulation by cyclothiazide of these two antagonist sites.

2 GYKI 100 μM, which is sufficient to eliminate virtually hippocampal kainate (100 μM) currents, failed to prevent access of cyclothiazide to its site of potentiation, and was unable to enhance removal of cyclothiazide potentiation. However, cyclothiazide reduced GYKI (30 μM) block from 84±8.3% to 38±12%, and slowed the onset of the block with a time course much faster than the time course for onset and offset of potentiation induced by cyclothiazide. Cyclothiazide had qualitatively similar effects upon antagonism by NBQX 1 μM.

3 Kainate activated desensitizing currents in dorsal root ganglion (DRG) neurones, which were unaffected by cyclothiazide. GYKI blocked these kainate currents with lower affinity (IC_{50} > 120 μM) than for hippocampal neurones (IC_{50} < 30 μM), and cyclothiazide did not affect GYKI antagonism.

4 Steady-state AMPA currents from homomeric GluRA-D_{flip} receptors in HEK 293 cells were dramatically potentiated (up to 216 fold) by cyclothiazide via reduction of desensitization. In contrast, kainate-gated currents in HEK 293 cells expressing GluR6R receptors exhibited pronounced desensitization that was unaffected by cyclothiazide. GYKI retains its inhibition at both recombinant AMPA and kainate receptors.

5 These results indicate that cyclothiazide allosterically influences two important antagonist sites on AMPA receptors. In addition, AMPA/kainate receptor subunit composition influences the affinity of GYKI for the receptor.

Keywords: AMPA receptor modulation; hippocampus; dorsal root ganglion; cyclothiazide; GYKI; thiazide; benzothiadiazide; benzothiadiazine

Introduction

The mammalian central nervous system is dependent to a great extent upon glutamatergic neurotransmitter systems for excitatory synaptic transmission. The glutamate receptors subserving this fast synaptic signaling are most likely composed of subunits from the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subset of glutamate receptors, designated GluRA-D (Keinanen *et al.*, 1990) or GluR1-4 (Hollmann *et al.*, 1989; Bettler *et al.*, 1990; Boulter *et al.*, 1990). The properties of individual subunits derived from studies of recombinant receptors suggest that functional diversity in native receptors may be dependent upon specific combinations of individual subunits. For example, the desensitization properties of AMPA receptors are influenced by alternative splicing involving a 38 amino acid sequence within the individual subunits called the flip/flop region (Sommer *et al.*, 1990), and by the presence of an arginine versus a glycine residue at the recently described 'R/G' site near the flip/flop region (Lomeli *et al.*, 1994). Differences in subunit composition may explain the significant differences in the rapidity and completeness of AMPA receptor desensitization observed in various native AMPA receptors (Trussell *et al.*, 1988; Tang *et al.*, 1989; Jonas & Sakmann, 1992; Raman & Trussell, 1992; Livsey *et al.*, 1993). In addition, strong evidence supports the existence of an intrinsic modulatory site on individual AMPA receptor subunits (Partin *et al.*, 1994; 1995) through which cyclothiazide acts to reduce desensitization of AMPA receptors (Yamada & Tang, 1993; Patneau *et al.*, 1993). Another modulatory site on the AMPA receptor was described by Zorumski and colleagues (1993), at which 2,3-benzodiazepines such as GYKI act to block non-competitively AMPA and kainate currents in hippocampal neurones. Interestingly, Zorumski and colleagues demonstrated that cyclothiazide and GYKI had concentration-dependent opposing actions, consistent with direct interaction at a common site (Zorumski *et al.*, 1993).

A number of observations make it attractive to think that there may be a specific modulatory site by which cyclothiazide exerts its effects upon AMPA desensitization and upon antagonists of the AMPA receptor, and evidence is accumulating that suggests that modulation via such a site may have clinically relevant implications (Moudy *et al.*, 1994; Zivkovic *et al.*, 1995). First, cyclothiazide is AMPA receptor selective (Partin *et al.*, 1993), and affects desensitization of flip splice variants more efficaciously and with higher affinity than flop splice variants (Partin *et al.*, 1994). Secondly, recent evidence suggests that the flip/flop sequence resides on an extracellular domain (Stern-Bach *et al.*, 1994), and functional GluR3-GluR6 chimeras involving the GluR3 N-terminal domain (probably the ligand binding domain) transposed onto a GluR6 channel/CO_2H terminal region require the inclusion of the putative extracellular loop containing the flip/flop region, supporting the view that this part of the protein is a necessary sequence involved with ligand binding. The reconstitution of

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cyclothiazide sensitivity in this chimera is consistent with the notion that the cyclothiazide site is within or near the flip/flop region. These results and the recent work of Mayer and colleagues establishing the requirement for Ser 750 in the flip/flop sequence for cyclothiazide sensitivity indicate that the flip/flop region is at least a functional component if not the location of the putative 'cyclothiazide site' (Partin *et al.*, 1995).

In this study we examined interactions between cyclothiazide and antagonists of AMPA and kainate receptors (GYKI and NBQX) using both native and recombinant receptors, to determine if cyclothiazide and GYKI acted at a common site on the AMPA receptor or if they interacted allosterically via two distinct sites to oppose the actions of each other. We utilized the AMPA receptor selective reduction of desensitization by cyclothiazide in order to separate the blocking effect of GYKI and we were also able to examine subunit selective antagonism by GYKI. As some of these AMPA/kainate receptor antagonists have neuroprotective actions in *in vivo* models of ischaemic neuronal injury (Sheardown *et al.*, 1990; Nellgard & Wieloch, 1992), regulation of their actions via these putative allosteric modulatory sites will have practical clinical relevance if such drugs are ultimately developed for use in man.

Methods

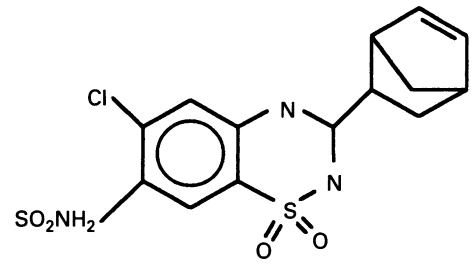
Cell cultures

Primary cultures of dissociated hippocampal neurones from P1 neonatal rat pups were prepared via methods originally described by Huettner and Baughman (1986), using slight modifications (Yamada & Tang, 1993). In brief, hippocampi from Sprague-Dawley P1 neonatal rat pups were removed, minced, and incubated with papain. The tissue was then mechanically dissociated by gentle trituration, centrifuged through a solution of albumin/trypsin inhibitor, and resuspended in growth medium. Neurones were plated on a layer of glial cells, which were prepared by preplating cortical glia from P1-4 rats 4 days earlier, and allowing them to grow to confluence. 5-Fluoro-2-deoxyuridine and uridine were added 2 days after the neurones were plated to prevent glial overgrowth. Cells were used 3–10 days after plating. Dorsal root ganglion (DRG) neurones were prepared in a similar fashion as described by Huettner (1990). The ganglia were removed from P1 rats and incubated in 8 mg ml^{-1} collagenase/dispase (Boehrenger Mannheim) for 30 min, triturated with successively smaller-tipped Pasteur pipettes, centrifuged and resuspended in growth medium without growth factors. DRG neurones were used 18–24 h after plating.

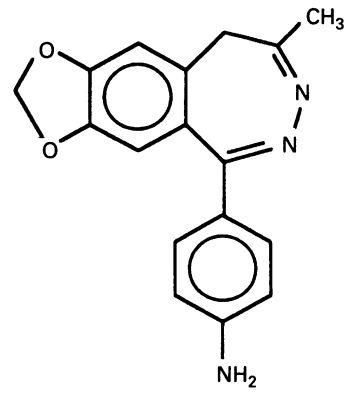
Expression of glutamate receptors in HEK 293 cell lines

Mammalian expression vectors containing CMV promoter sequences and full length cDNAs for GluRA, GluRB, GluRC, GluRD and GluR6R were kindly provided by H. Monyer and P. Seeburg. HEK 293 cells were transfected by a 15 h exposure to Lipofectace (Gibco). pRc/CMV (Invitrogen) was co-transfected with the AMPA receptor to provide a selectable marker. NBQX 10 μM was present during the transfection and in all subsequent steps to prevent activation of expressed receptors by glutamate in the medium. Selection of stably transfected colonies was achieved in 500 μg ml^{-1} G418; resistant colonies were screened for functional GluR A, C and D receptors using fura-2 microfluorimetry. Lines in which AMPA and/or kainate produced increases in intracellular calcium concentration were screened by immunocytochemistry to determine the percentage of cells expressing the receptor, and the level of expression. GluRB lines were screened solely by immunocytochemistry. For each receptor, the line with the strongest expression in >70% of the cells was selected for further study. These lines were maintained in 10 μM NBQX and 500 μg ml^{-1} G418.

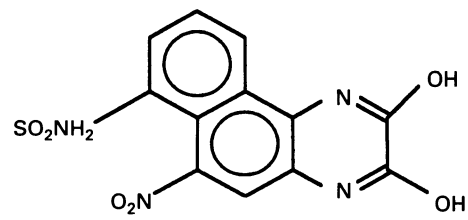
For transient transfections, HEK 293 cells were plated on poly-D-lysine and laminin coated 35 mm dishes and grown to ~15% confluence. Immediately before transfection the cells were washed twice in OptiMEM (Gibco) to remove all traces of antibiotics and serum. The supercoiled plasmid DNA (2 μg ml^{-1} final concentration) and Lipofectamine (24 μg ml^{-1} final concentration, Gibco) solution was then added to the cells for 2 h. Occasionally transfection was done by electroporation, using the Biorad Gene Pulser (equipped with a capacitance extender) at 350 mV, 960 μF. After transfection the cells were washed and placed in growth medium (MEM + 10% FBS) containing 10 μM NBQX. Transfection rates were typically 40–50%. Cells were used 24–72 h after the start of transfection.



Cyclothiazide



GYKI 52466



NBQX

Figure 1 Chemical structures of drugs whose interactions were examined in this study. Cyclothiazide is a benzothiadiazide which reduces AMPA desensitization (Yamada & Tang, 1993), GYKI 52466 is a non-competitive AMPA/kainate antagonist (Tarnawa *et al.*, 1989; Zorumski *et al.*, 1993; Donevan & Rogawski, 1993), NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline) is a competitive AMPA/kainate antagonist (Sheardown *et al.*, 1990).

Electrophysiology

The external recording solution used with hippocampal neurones contained (in mM): NaCl 140, KCl 3.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, HEPES 10, phenol red 10 mg l^{-1}, tetrodotoxin 0.6 μM and MK-801 20 μM; pH 7.3. The external recording solution used with HEK 293 cells was identical except tetrodotoxin and MK-801 were excluded. The internal recording solution contained (in mM): CsCl 130, TEACl 10, HEPES 10, EGTA 1.1 and glucose 5.5; pH 7.2. Glutamate agonists were dissolved directly into the external recording solution or were made from aqueous stock solutions. Stock solutions of cyclothiazide and GYKI-52466 were made in dimethylsulphoxide (DMSO) and diluted. The final DMSO concentration was usually 0.1–0.76% by volume, except in experiments with DRG neurones in which 3.6% DMSO was used because of the limited solubility of GYKI. Appropriate amounts of DMSO were added to all recording solutions to provide equivalent final concentrations for a given experiment. Recordings were performed at room temperature. Patch electrodes were pulled in three stages (Sutter Instruments, Novato, California) and had d.c. resistances of 5–10 MΩ. These electrodes were connected to a patch amplifier in voltage-clamp mode using the whole-cell configuration (Hamill *et al.*, 1981). Approximately 60–80% of the series resistance was compensated for. The holding potential was −60 mV unless indicated otherwise.

Drug application

Cells were plated onto 35 mm plastic dishes, which were placed on an inverted microscope stage. Continuous perfusion of the dish with drug-free recording solution was accomplished via gravity flow at a rate of 2–3 ml min^{-1}. The cell recorded from was also locally perfused via a large-bore fused silica-quartz tube (internal diameter 320 μm) connected to four separate reservoirs, each with a separate solenoid valve to control its flow. Only one valve could be opened at a time. Agonists without or with drugs were applied via a second flow tube, also connected individually to four separate reservoirs. Agonist was applied by simultaneously switching off the local wash reservoir while opening the agonist reservoir. Flow occurred via gravity. The rapidity of agonist application with this method was estimated by measuring the holding current change in voltage-clamp mode in response to application of solutions with different osmolarity. The holding current changed with a monoexponential risetime of less than one millisecond.

Data are presented as means ± 1 s.d. Two-tailed t test was used to assess differences, which were considered statistically significant if $P<0.05$. Actual P values are usually shown in parentheses when significant, e.g., ($P=0.001$) or by 'N.S.', when not significant.

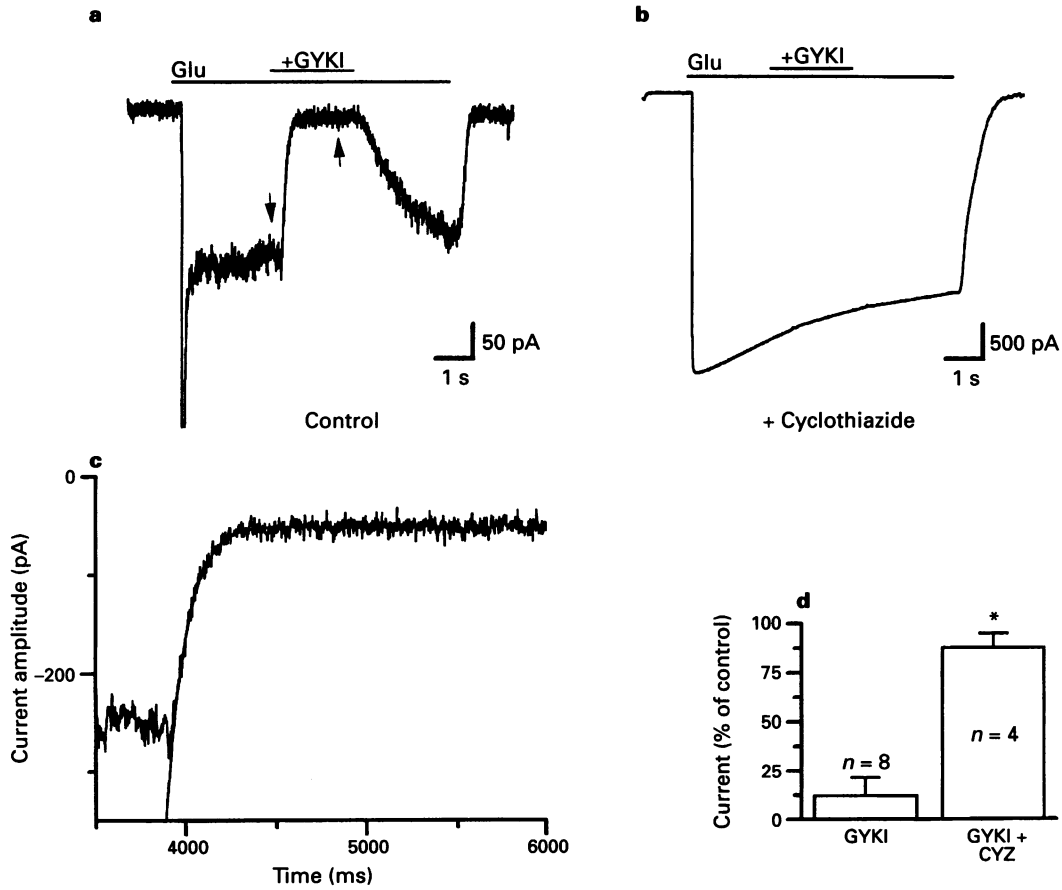


Figure 2 Steady-state L-glutamate (Glu) activated currents in hippocampal neurones are blocked by GYKI and that block is almost completely eliminated by cyclothiazide (CYZ). (a) Glu (1 mM) produced a large transient current that is partially truncated to better illustrate the block of the steady-state current by GYKI 30 μM. The short bar above the traces indicates when the solution was switched from Glu to Glu+GYKI. The extent of GYKI block is assessed by showing the current amplitude in GYKI (measured at up arrow) as a percentage of the control current (measured at down arrow). (b) In the same cell, cyclothiazide 100 μM almost eliminated desensitization, potentiated the current (note scale bars), and the block of GYKI was virtually eliminated. (c) The onset of the block was best fit by a two exponential function, the major component having a time constant of 100 ms. (d) Cumulative data from 8 cells indicate that GYKI produced 88 ± 9.2% block of the steady-state Glu current in the absence of, and about 12 ± 7.4% block in the presence of CYZ (*$P<0.0001$).

Results

The chemical structures of cyclothiazide, GYKI, and NBQX are shown in Figure 1. Cyclothiazide is a member of a group of drugs called benzothiadiazides, which reduce AMPA desensitization (Yamada & Tang, 1993), GYKI is a non-competitive AMPA/kainate antagonist (Tarnawa *et al.*, 1989; Zorumski *et al.*, 1993; Donevan & Rogawski, 1993), and NBQX is a competitive AMPA/kainate antagonist (Sheardown *et al.*, 1990). Figure 2 shows the effects of cyclothiazide and GYKI on whole-cell currents gated by L-glutamate (Glu) in a cultured hippocampal neurone. The large inward current produced by a concentration jump to 1 mM Glu at −60 mV rapidly desensitized to a small steady-state value. After desensitization reached this steady-state level, another concentration jump to Glu plus 30 μM GYKI shows that GYKI blocked the steady-state current to 12.3±9.2% ($n=8$) of the control steady-state current with a time constant of onset of block of approximately 100 ms. The fact that GYKI blocked the steady-state current at a saturating concentration of Glu is consistent with its non-competitive mechanism of antagonism. Cyclothiazide 100 μM almost completely eliminated the desensitization and eliminated the blockade of the current by GYKI (88±7.4% of control, $n=4$, $P<0.0001$). These opposing actions of cyclothiazide and GYKI are similar to those initially characterized by Zorumski and colleagues (1993).

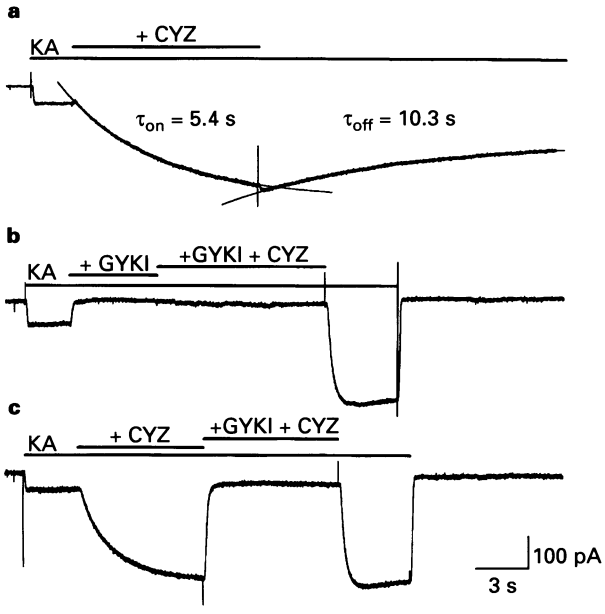


Figure 3 The onset of potentiation by cyclothiazide of kainate currents was much slower than the onset of the blocking effect of GYKI. (a) Kainate 100 μM (KA) produced a non-desensitizing current in a hippocampal neurone at −30 mV. A solution jump to kainate plus 100 μM cyclothiazide (CYZ) produced a small block followed by slow potentiation of the current. A single exponential curve fitted to the current trace has a time constant of 5.4 s. When the cyclothiazide was removed, the block was relieved followed by relief of potentiation that was slower than its onset, a single exponential curve fitted to the relief of potentiation has a time constant of 10.3 s. (b) In another cell the kainate current was almost completely blocked by 100 μM GYKI, and 100 μM CYZ minimally counteracted the block due to GYKI. However, the potentiating effect of CYZ was present upon removal of both GYKI and CYZ, indicating that CYZ accessed its site of potentiation in the presence of GYKI. Holding potential −30 mV. (c) Same cell as in (b), when CYZ was applied before GYKI, there was potentiation, and GYKI was unable to block the current completely. Although GYKI appeared to eliminate CYZ potentiation, upon removal of both GYKI and CYZ the presence of the same degree of potentiation suggests that CYZ remains at its site of action for potentiation unaffected by the presence of GYKI. Vertical transients are an artifact produced by solenoid valve actuation.

The reduction of glutamate desensitization (Yamada & Tang, 1993) and potentiation of kainate currents (Patneau *et al.*, 1993) induced by cyclothiazide require several seconds to develop, and recovery from these effects takes even longer. Mayer and colleagues (Patneau *et al.*, 1993) demonstrated a single exponential time constant of 34 s for the removal of the potentiating effect of cyclothiazide upon kainate currents in hippocampal neurones. We took advantage of the slow onset and offset kinetics of cyclothiazide to address whether or not the interaction between GYKI and cyclothiazide was directly competitive.

In Figure 3 kainate was applied to a hippocampal neurone and a non-desensitizing current was obtained. When cyclothiazide was applied there was a brief reduction in the kainate current, followed by slow potentiation of the current as previously reported by Patneau *et al.* (1993). Upon removal of cyclothiazide, there was brief potentiation of the kainate current followed by a slow recovery from the potentiating effect. The time constants for a single exponential fit to the onset and recovery of the effects of cyclothiazide were variable but always several seconds ($\tau_{on}=7.3\pm3.1$ s, $n=12$; $\tau_{off}=17.3\pm7.8$ s, $n=11$). GYKI 100 μM almost completely blocked the kainate current, and when the solution was switched to GYKI+cy-

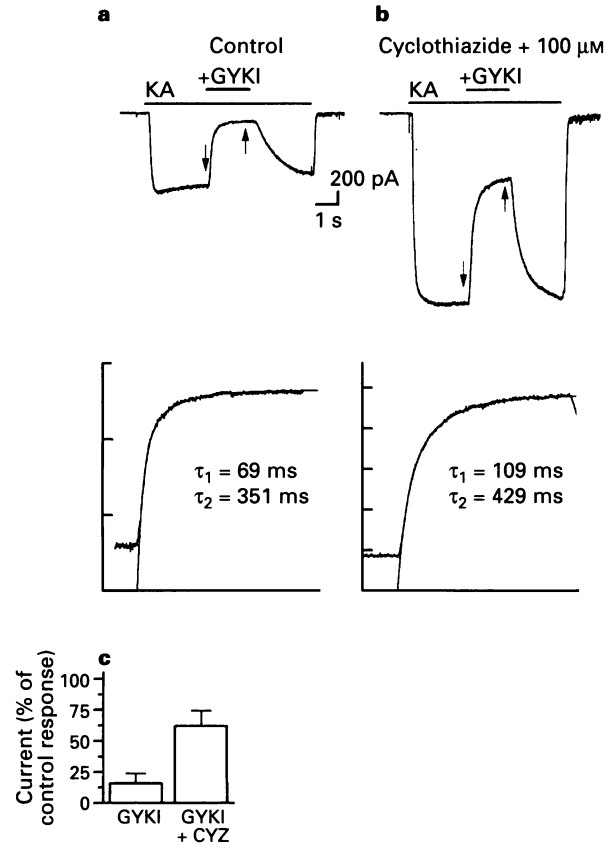


Figure 4 GYKI blockade of kainate (KA) currents in hippocampal neurones was reduced and slower in onset in the presence of cyclothiazide. (a and b) Top similar solution jumps as shown in Figure 2 in another representative cell showed that GYKI 30 μM blocks the currents produced by 100 μM KA in the absence and presence of cyclothiazide. GYKI block was quantitated by measuring the current amplitude in GYKI (at up arrow) and comparing it to the control current (measured at the down arrow). (b) Cyclothiazide (CYZ) 100 μM potentiated the KA current, but in addition the extent of block by GYKI was reduced (cumulative data from 9 cells are shown in c), and the onset of the block was slower. The insets below the current traces are magnified views of the onset of GYKI block shown in the top panels, including the curves obtained on fitting the data with a double exponential function (y-axis tick marks are at 500 pA intervals in both insets). The two time constants for the fitted curves are shown.

clothiazide there was only a slight reduction of GYKI block (Figure 3b). In contrast, upon removal of both GYKI and cyclothiazide, the potentiating effect of cyclothiazide was fully intact, unaffected by the presence of a complete blocking concentration of GYKI, and not exhibiting its usually slow onset of potentiation as shown in Figure 3a. When cyclothiazide was applied before 100 μM GYKI as shown in Figure 3c, the kainate current was potentiated by cyclothiazide, and the potentiated current was significantly blocked by GYKI. Although it appears that the effect of cyclothiazide was completely eliminated by GYKI, because GYKI blocked the kainate current to approximately its control magnitude, upon removal of both GYKI and cyclothiazide, the full potentiating effect of cyclothiazide was fully intact. Therefore, GYKI concentrations sufficient to block most or all of the control or cyclothiazide-potentiated kainate current failed to reduce potentiating effects of cyclothiazide, arguing against a competitive interaction between the two drugs.

We hypothesized that there must be an allosteric interaction between GYKI and cyclothiazide that would explain cyclothiazide's reduction of GYKI antagonism shown in Figure 2, and sought to quantitate the effects of cyclothiazide upon the kinetics of antagonism by GYKI of AMPA receptor-gated currents. We studied whole-cell currents gated by the non-desensitizing agonist kainate first against GYKI alone and then in the presence of 100 μM cyclothiazide. Figure 4 shows a representative hippocampal neurone in which kainate activates a non-desensitizing inward current that is blocked by 30 μM GYKI. Block was quantitated by measuring the current amplitude in the presence of GYKI as a percentage of the current amplitude without GYKI (see Figure 4 legend). GYKI blocked kainate currents to $16 \pm 8.3\%$ of control ($n=9$, Figure 4c). This degree of blockade is comparable to that observed by Zorumski and colleagues (1993) and Donevan and Rogawski (1993), both of whom quantitatively determined the IC_{50} of GYKI to be of 14 and 11 μM, respectively. In these same 9 cells cyclothiazide potentiated the control kainate current in every cell to varying degrees (1.2 to 4.6 fold, mean 2.6 ± 1.0, $n=9$). Notably, in the presence of cyclothiazide, GYKI blocked the kainate current to just $62 \pm 12\%$ of the control response ($P<0.00001$), and this proportion of block was similar regardless of the amount of current potentiation by cyclothiazide. The time course of inhibition by GYKI of kainate currents was well fit by a double exponential function, and the time constants of onset of block were 94 ± 18 ms and 388 ± 228 ms ($n=9$). The initial component is comparable to the time constant for onset of GYKI block of 97 ms obtained by Donevan and Rogawski (1993). In the presence of 100 μM cyclothiazide the time constants for block were prolonged, 164 ± 39 and 861 ± 455 ms, respectively, and the fits closely approximated the data. Both differences are statistically significant ($P<0.001$ and $P=0.01$, respectively).

Similar experiments were performed using 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) 1 μM instead of GYKI, in order to determine if cyclothiazide affected the onset kinetics and the magnitude of NBQX block. Figure 5 shows an example of whole-cell kainate currents in a cultured hippocampal neurone in which 1 μM NBQX produced block ($6.2 \pm 4.4\%$ of control, $n=7$). The time course of onset of block consisted of a predominant fast component that had a time constant around 35 ms, and one or two slower components. In 100 μM cyclothiazide, there was a 2.2 ± 1.0 fold potentiation of the control kainate current (range 1.4–4.1 fold, $n=7$), and the block was reduced ($42 \pm 16\%$ of control, $n=7$, $P=0.001$). Qualitatively there was always clear prolongation of the onset of NBQX block in the presence of cyclothiazide as exemplified in Figure 5 in which a good fit was obtained with a two exponential function. However, sometimes the onset of block was not fit well by a double exponential function, preventing quantitative comparison of the effect of cyclothiazide upon NBQX in the same fashion as was done for GYKI.

Because DRG neurones possess kainate-preferring receptors, possibly GluR5±KA-2 (Bettler *et al.*, 1990; Herb *et al.*, 1992), we examined GYKI antagonism without and with cyclothiazide in DRG neurones. Identical solution jumps were performed as shown previously in Figure 4, except that higher concentrations of GYKI were used (40–400 μM). In contrast to Glu or kainate currents from hippocampal neurones, in which constant steady-state currents were reached before making a solution exchange to agonist+GYKI, kainate currents in DRG neurones continued to desensitize during the solution jump to kainate+GYKI, which would artificially exaggerate the magnitude of GYKI block. Therefore, in order to account for ongoing desensitization, the desensitizing kainate current was fitted by a double exponential function which was extrapolated into the part of the tracing during kainate+GYKI application. GYKI block was quantitated as the current amplitude in GYKI as a percentage of the control current which was measured from the extrapolated current trace (see Figure 6a, right). Cyclothiazide (100 μM) did not reduce kainate desensitization or potentiate kainate currents in DRG neurones, consistent with their probable composition of kainate-preferring, cyclothiazide insensitive receptors (Wong & Mayer, 1993). Moreover, cyclothiazide had no effect on GYKI block (Figure 6b). Although traces are not shown, 40 and 120 μM GYKI (40 μM without and with cyclothiazide) were also studied and the cumulative data from 4 cells tested

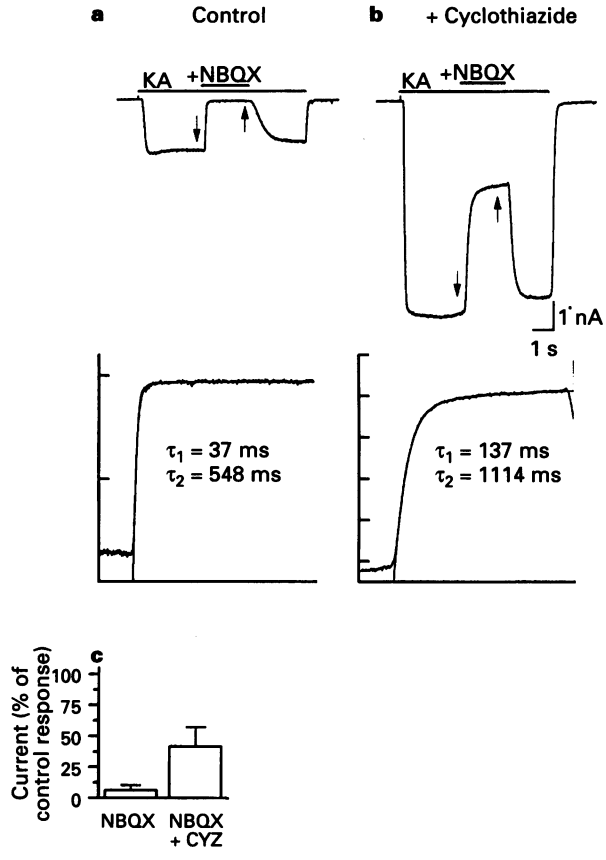


Figure 5 NBQX blockade of kainate currents in hippocampal neurones was reduced and slower in onset in the presence of cyclothiazide. (a) NBQX 1 μM blocked the current from 100 μM kainate (KA). NBQX block was quantitated by measuring the current in NBQX (at the up arrow) and comparing it to the control current (at the down arrow). (b) The same solution jumps were made as in (a) on the same cell, except that cyclothiazide 100 μM was included in all of the solutions. The extent of the block was reduced in cyclothiazide (CYZ) (summarized for 7 cells in c), and in addition the onset of the block was slower. The insets below the current traces are magnified views of the onset of NBQX block for traces in (a) and (b), including the curves fitting the data with a double exponential function (the y-axis tick marks are 1 nA for both insets). The two time constants for the fitted curves are shown.

with these three concentrations of GYKI are shown in Figure 6b. GYKI 40, 120 and 400 μM blocked kainate currents to 80 ± 3.9, 71 ± 7.3, and $23 \pm 9.7\%$ of control respectively ($n=4$). These three GYKI concentrations were also tested against peak kainate currents in 6 other DRG neurones (Figure 6c); the cumulative data demonstrate that peak current amplitudes were reduced to 72 ± 11, 57 ± 10, and $27 \pm 6.4\%$ of control by 40, 120, and 400 μM GYKI respectively (Figure 6d). These results indicate that GYKI blocks the peak and the desensitizing kainate current with an IC_{50} between 120 and 400 μM, roughly 10 times or greater than the IC_{50} of GYKI against kainate currents in hippocampal neurones. This is comparable to the IC_{50} of >200 μM found in DRG neurones by Wilding and Huettner (Huettner & Wilding, 1995).

The effects and interactions between cyclothiazide and GYKI exhibit significant differences between hippocampal AMPA receptors and DRG kainate receptors, presumably due to differing physiological properties of specific subunits comprising the native receptors. Therefore, we tested whether or not similar differences between cyclothiazide and GYKI interactions were exhibited by recombinant homomeric AMPA and kainate receptors. Figure 7 shows AMPA or kainate gated whole-cell currents from single HEK 293 cells expressing homomeric channels comprised of GluRA-D_{flip} or GluR6R subunits, demonstrating the selective action of cyclothiazide upon AMPA receptor desensitization (cumulative data summarized in Table 1). Glu 1 mM produced barely detectable currents from all of the AMPA receptors except for D_{flip}. The figure shows examples from cell lines, but the results were comparable with transiently expressed receptors (not shown). Cyclothiazide caused unequivocal reduction of desensitization and potentiation of current in all cells expressing AMPA subunits. Assessment of the effect was determined by comparing the steady-state current without and with cyclothiazide. The range of potentiation of AMPA currents in GluRA-D_{flip} expressing cells was 4.5–216 fold (Table 1); desensitization was essentially eliminated by 10 μM cyclothiazide. In contrast, GluR6R expressing cells exhibited desensitizing responses to kainate, unaffected by the relatively high concentration of 100 μM cyclothiazide (Figure 7), and AMPA failed to gate a detectable current even in the presence of 100 μM cyclothiazide (not shown). Cyclothiazide actually blocked the peak kainate current to $86 \pm 8.7\%$ of the control response ($P<0.001$, $n=8$).

We than examined whether or not GYKI blocked GluRA-D_{flip} versus GluR6R receptors with differing affinity or possibly some receptor subunits but not others, and found that GYKI blocked both AMPA and kainate currents in $GluRC_{flip}$, $GluRD_{flip}$ and GluR6R receptors (Figure 8a and b). As shown in Figure 8c, 100 μM GYKI depressed kainate currents in GluR6R expressing HEK 293 cells to $58 \pm 12\%$ of control ($P<0.001$, $n=6$). In 3 of these cells 100 μM cyclothiazide failed to reduce GYKI block ($60 \pm 10\%$ of control, NS), indicating that the two drugs do not compete against each other. In prior studies (not presented here) we estimated a lower IC_{50} for GYKI, but did not control for DMSO concentration or completely eliminate low levels of kainate, both of which would overestimate the antagonism by GYKI. Therefore, in other experiments we preincubated cells with ConA 0.3 mg ml^{-1} to reduce GluR6R receptor desensitization and more clearly demonstrate GYKI antagonism. Under these conditions, only 100 μM GYKI unequivocally depressed kainate currents ($82 \pm 6.3\%$ of control, $P<0.001$, $n=6$), and did so less than in the absence of ConA (Figure 8c).

Discussion

GYKI and cyclothiazide interactions at hippocampal AMPA receptors

Our initial observations that GYKI and cyclothiazide had opposing actions upon AMPA and kainate gated currents in

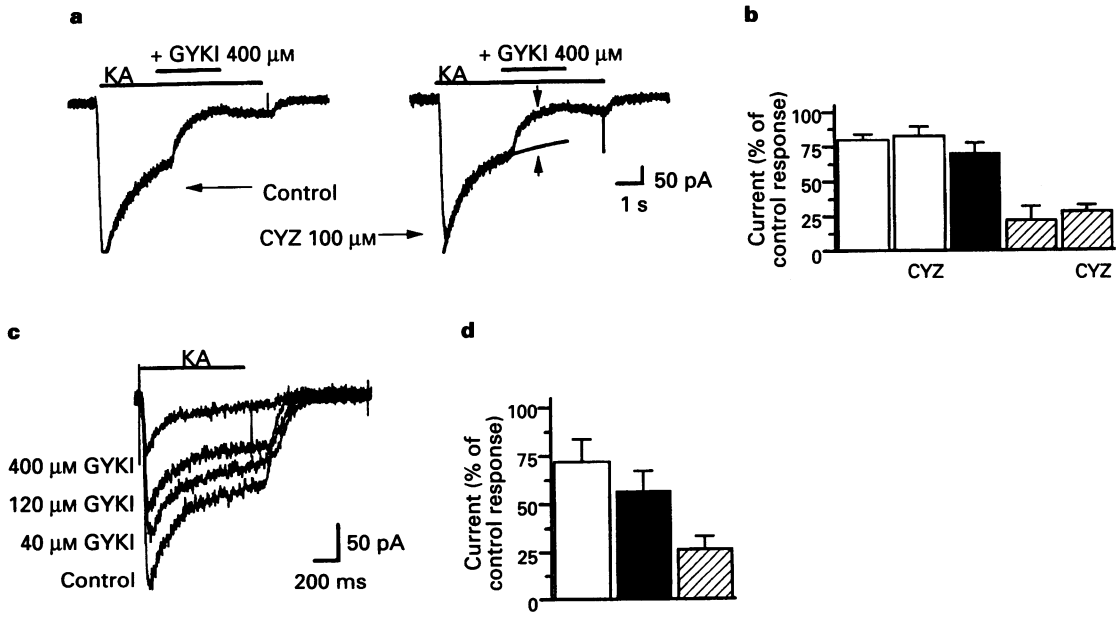


Figure 6 The concentration-dependence of GYKI antagonism of kainate (KA, 100 μM) currents was different for dorsal root ganglion (DRG) neurones compared to hippocampal neurones. (a) An example of KA currents in a typical cultured DRG neurone in which concentration jumps from KA to KA+GYKI 400 μM were performed in the absence (left) or presence (right) of cyclothiazide 100 μM (CYZ) as shown in Figure 4. The method of measuring the block is shown on the right. A curve fitted to the desensitizing KA current was extrapolated into the portion of the tracing during which GYKI was being applied. GYKI block was quantitated by measuring the current amplitude in GYKI (at the down arrow) and comparing it as a percentage of the control response, which was measured from the extrapolated fitted curve (at the up arrow). CYZ did not reduce KA desensitization or potentiate KA currents in DRG neurones, and the block by GYKI was unaffected by CYZ. (b) A summary of data from 4 cells; 400 μM GYKI (hatched columns) blocked KA currents by about 70% whereas 40 (open columns) and 120 (solid column) μM had little effect indicating that the IC_{50} of GYKI against KA currents in DRG neurones was between 120 and 400 μM. (c) Preapplication of 3 concentrations of GYKI on a typical cell demonstrated a similar concentration-dependence upon block of the peak KA currents, summarized for 6 cells in (d). (For key to shading of columns see (b)). The IC_{50} for GYKI was between 120 and 400 μM.

hippocampal neurones, were consistent with direct competition at a common site we called the 2,3-benzodiazepine recognition site (Zorumski *et al.*, 1993). Consistent with our previous study, we found that 30 μM GYKI almost completely blocked the steady-state current produced by 1 mM L-glutamate (Glu), and the blockade was virtually eliminated by 100 μM cyclothiazide. Notably, 1 mM Glu is at least 30 times the steady-state EC_{50} for Glu (Zorumski & Thio, unpublished observation), underscoring the non-competitive mechanism of antagonism of GYKI (Tarnawa *et al.*, 1989; Zorumski *et al.*, 1993; Donevan & Rogawski, 1993). This result indicated that enhancement by cyclothiazide of Glu affinity (Yamada & Tang, 1993; Patneau *et al.*, 1993) was unlikely to account for its ability to reduce GYKI antagonism. Moreover, the onset time course of GYKI blockade had a time constant of about 100 ms, about 5 times slower than the estimated time to complete whole-cell solution exchange (unpublished observation), indicating that our time course measurements for GYKI block should not be limited by the speed of our perfusion system.

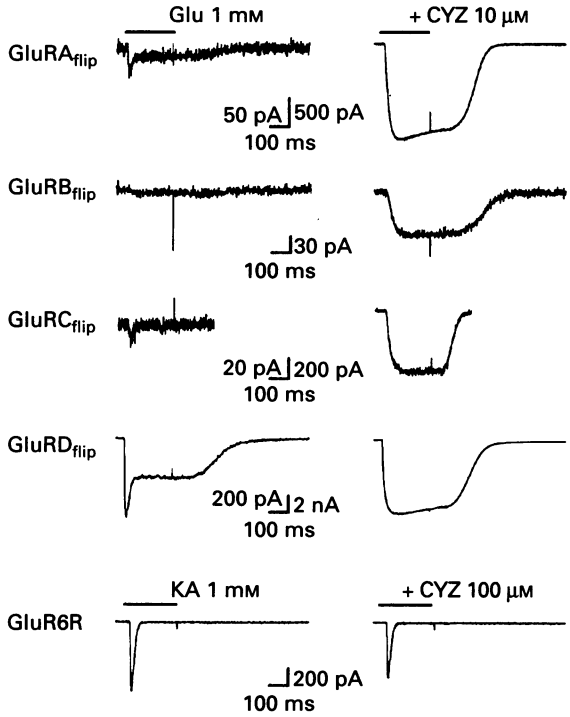


Figure 7 Homomeric recombinant GluRA-D_{flip} receptors expressed in HEK 293 cells exhibited desensitization that was virtually eliminated by cyclothiazide (CYZ), while homomeric recombinant GluR6R receptors exhibited desensitization that was unaffected by CYZ. Representative traces are shown from 4 HEK 293 cells from lines that possessed stable expression of the GluR receptors indicated on the left after application of L-glutamate (Glu) 1 mM without and with CYZ 10 μM. The bottom pair of traces is from an HEK 293 cell transiently expressing GluR6R to which 1 mM KA without and with CYZ 100 μM was applied. No potentiation or effect upon desensitization was observed, but instead there was a small reduction of the peak current.

The most direct evidence against competition between GYKI and cyclothiazide are the results from sequences of long applications of the two drugs alone and in combination. We showed that 100 μM GYKI produced nearly complete block of kainate currents, which was essentially unchanged when solutions were switched to GYKI plus 100 μM cyclothiazide. Then, in spite of the persistent GYKI block in the presence of both drugs, upon their removal the kainate current was potentiated as if cyclothiazide had been applied alone, indicating that although the presence of GYKI appeared to prevent the effect of cyclothiazide, in reality cyclothiazide was still able to access its site of action and produce its full potentiating effect, without requiring its usually slow onset of action. Conversely, when kainate currents were potentiated by cyclothiazide, 100 μM GYKI blocked essentially all of the potentiation in spite of the continued presence of cyclothiazide, leaving a current amplitude similar to the control response. Although the cyclothiazide effect was apparently completely reversed, upon removal of GYKI and cyclothiazide full potentiation of the kainate current was observed without exhibiting the slow onset of potentiation that would be expected if GYKI had to come off and cyclothiazide had to bind to a common site of action before potentiating the kainate current.

Although our results argue against a direct interaction between GYKI and cyclothiazide, we have clearly shown that cyclothiazide reduces the proportion of Glu and kainate currents blocked by GYKI, which is independent of the magnitude of cyclothiazide potentiation. We also demonstrated that cyclothiazide affected the kinetics of GYKI block by assessing the time course of GYKI block in the presence of cyclothiazide, exploiting the disparity between the onset/offset of the potentiation by cyclothiazide of kainate currents (tens of seconds) and the onset of GYKI block (hundreds of milliseconds). The time course we determined for GYKI block of

Table 1 Glutamate or AMPA gated currents from HEK 293 cells transiently or persistently expressing homomeric GluRA-D_{flip} receptors exhibited desensitization that was reduced or eliminated by cyclothiazide

GluR subunit expressed	*[Agonist]*	*[CYZ]*	*Potentiation, in multiples of control response*	*Number of cells*
A, stable	Glu 1mM	10 μM	102±42	3
B, stable	Glu 1mM	10 μM	27±34	4
C, stable	Glu 1mM	10 μM	216±131	4
D, stable	Glu 1mM	10 μM	16	2
A	AMPA 100 μM	100 μM	17±3.5	5
B	AMPA 100 μM	100 μM	4.6±0.77	5
C	AMPA 100 μM	100 μM	12	2
D	AMPA 100 μM	10 μM	4.5±3.4	6
GluR subunit expressed	*[Agonist]*	*[CYZ]*	*Inhibition (% of control response)*	*Number of cells*
6R	KA 300 μM or 1 mM	100 μM	86±8.7	8

Abbreviations used: Glu = L-glutamate; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; KA = kainate; CYZ = cyclothiazide.

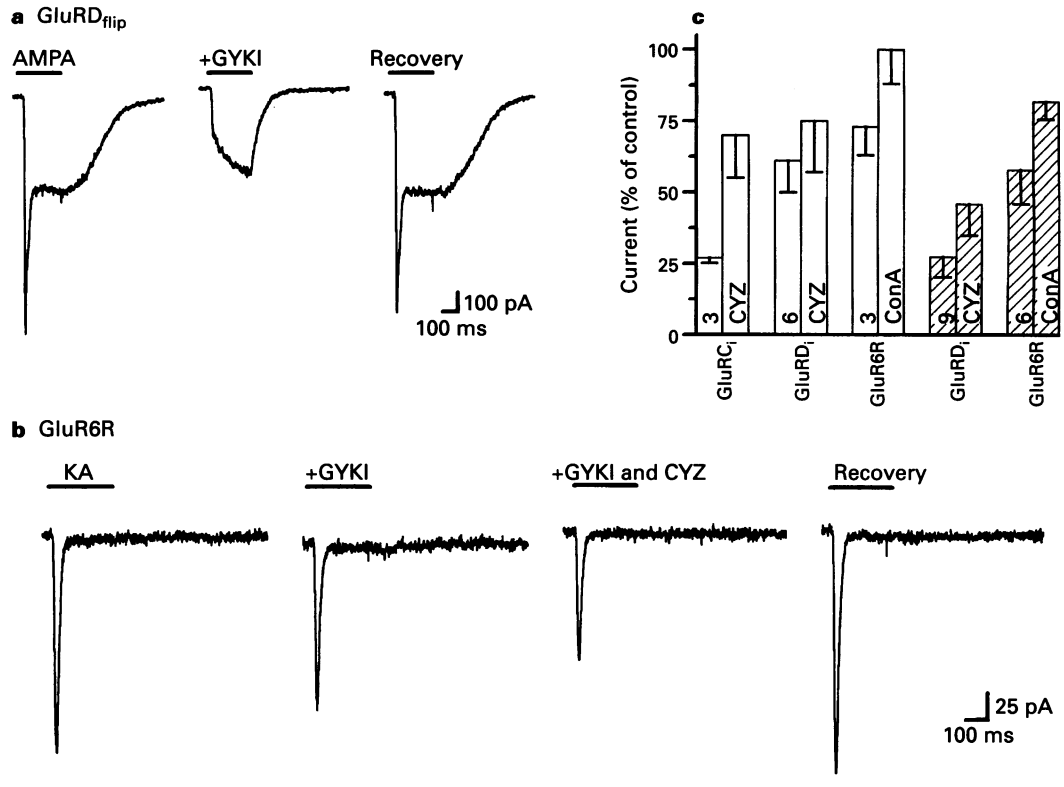


Figure 8 AMPA and kainate currents from GluRC$_{flip}$, GluRD$_{flip}$ and GluR6R recombinant receptors were blocked by GYKI. (a) AMPA 300 μM produced a desensitizing current in a GluRD$_{flip}$ expressing HEK 293 cell, and GYKI 100 μM blocked most of the peak current. In this cell the GYKI was applied only prior to the AMPA application, but the magnitude of reduction of the peak current was not greatly affected by preapplication versus preapplication plus coapplication of GYKI. The data are therefore combined in (c). (b) GYKI 15 μM produced a small block of the peak kainate (KA, 300 μM) current in HEK 293 cells expressing GluR6R, and this small degree of block was not overcome by cyclothiazide (CYZ) 100 μM. (c) Cumulative data exemplified in (a) and (b). GYKI 15 (open columns) and 100 (hatched columns) μM blocked peak AMPA/KA currents in homomeric GluRC$_{flip}$, GluRD$_{flip}$ and GluR6R receptors. The second column of each pair indicates the extent of blockade of steady-state currents when desensitization was eliminated by CYZ 100 μM for GluRC$_{flip}$ and D$_{flip}$, or concanavilin A 0.3 mg ml^{-1} (ConA) for GluR6R receptors. Number of cells indicated within columns. The estimated IC_{50} for GYKI is between 15–100 μM for GluRC and D, while it is 100 μM or greater for GluR6R.

hippocampal kainate currents was comparable to that found by Donevan and Rogawski (1993) (τ_{onset} = 97 ms), except that in 8 of 9 cells a better fit to our data was obtained by a two exponential function. The two exponential fit was retained in cyclothiazide, which produced significant increases of both time constants (roughly by 100%). We did not observe a slow component corresponding to the offset time of cyclothiazide constant of 17, which should have been observed if cyclothiazide had to dissociate from a common site before GYKI could exert its antagonism. Therefore, we concluded that cyclothiazide allosterically interacts with GYKI, both reducing the extent and slowing the onset of its block.

We also demonstrated that cyclothiazide had qualitatively similar effects upon the onset and extent of block of kainate gated currents by the competitive antagonist NBQX. Because NBQX competes directly at the ligand binding site, cyclothiazide necessarily acts allosterically to hinder NBQX blockade, perhaps in part via its ability to increase kainate affinity (Patneau *et al.*, 1993). In summary, our data suggest that cyclothiazide exerts allosteric modulatory effects upon two important antagonist sites on the AMPA receptor, slowing the onset of action and reducing the extent of blockade by AMPA antagonists with both competitive and non-competitive mechanisms of action.

GYKI antagonism at DRG kainate receptors

Certain DRG neurones possess kainate-preferring receptors that exhibit pronounced desensitization (Huettner, 1990), unaffected by cyclothiazide (Wong & Mayer, 1993). We also found that cyclothiazide did not affect the desensitization of DRG kainate currents. Initially, when we believed that GYKI and cyclothiazide competed for a common site, we hoped that without the potentiating effects of cyclothiazide we could easily demonstrate that cyclothiazide competitively eliminated the antagonism by GYKI. Instead, we found that cyclothiazide had no effect on the antagonism by GYKI, unequivocally demonstrating that on DRG kainate receptors the sites of action for cyclothiazide and GYKI are distinct and independent of each other. However, one cannot conclude that AMPA and kainate GYKI sites are homologous because our data and the data from others indicate that GYKI has a higher affinity for AMPA receptors than kainate receptors. Our data indicate that the IC_{50} for GYKI at DRG kainate receptors is between 120 and 400 μM, at least 10 fold higher than we estimated for hippocampal AMPA receptors, which has been shown to be between 9.8 and 14 μM (Zorumski *et al.*, 1993; Donevan & Rogawski, 1993; Paternain *et al.*, 1995). Huettner and Wilding (1995) reported comparable IC_{50} differences for GYKI between cortical AMPA receptors and DRG kainate receptors. Moreover, Lerma and colleagues (Paternain *et al.*, 1995) have shown that certain hippocampal neurones have a component of kainate gated current that exhibits desensitization and are completely insensitive to GYKI 53655 or relatively insensitive to GYKI 52466 (roughly to the same degree as we observed in the DRG and recombinant GluR6R receptors). We believe these are important observations, because they imply that the subunit composition of AMPA/kainate receptors influences the affinity of GYKI, ultimately requiring that pharmacological uses of GYKI compounds must take into account the subunit composition of the targeted receptors. A favourable consequence is that specific subunits might be

targeted by antagonist drugs, potentially resulting in more selective and potent drugs with fewer unwanted side effects.

GYKI and cyclothiazide effects upon recombinant AMPA/kainate receptors

In HEK 293 cells, cyclothiazide reduced desensitization of recombinant GluRA-D_{flip} AMPA receptors, but had no effect upon recombinant GluR6R receptor desensitization as previously shown by Mayer and colleagues (Partin *et al.*, 1994). An interesting observation was that in 3 of our 4 stable AMPA expressing cell lines almost undetectable AMPA receptor gated currents were enormously potentiated by cyclothiazide, suggesting that high levels of homomeric receptor expression were hidden by their desensitized state, not necessarily because of diminished expression or inefficient receptor-channel assembly. In fact, our screening procedure for stable expression utilized the potentiation by cyclothiazide of AMPA or kainate stimulated intracellular calcium increases detected by Fura-2 microfluorimetry. We observed that cyclothiazide reduced the extent of GYKI blockade of AMPA currents from GluRC and D (flip) expressing cells, similar to our results from hippocampal AMPA receptors and as demonstrated in recombinant GluR1, GluR2, and GluR1 + GluR2 receptors expressed in *Xenopus* oocytes (Sharp *et al.*, 1994). Because cyclothiazide acts upon homomeric channels composed of any one of the GluRA-D_{flip} subunits, its site of action must be an intrinsic element of the individual AMPA receptor subunit. Moreover, recent data suggest that the flip/flop region is likely to be an extracellular domain (Stern-Bach *et al.*, 1994) and that within this domain reside critical elements necessary for the effect of cyclothiazide against AMPA desensitization (Partin *et al.*, 1995).

We found that GYKI blocked currents in both AMPA and kainate receptor expressing HEK 293 cells, but that the higher concentrations were required to block kainate receptors. When we preincubated GluR6R expressing HEK 293 cells with ConA to reduce the confounding effect of desensitization, only 100 μM GYKI produced unequivocal blockade of kainate currents, and blockade was unaffected by cyclothiazide, similar to our results from DRG neurones. The ability of ConA to reduce GYKI blockade at kainate receptors may be analogous to the effect of cyclothiazide upon GYKI antagonism at AMPA receptors, but we also suspect that very low concentrations of kainate may desensitize receptors leading to overestimation of the blocking ability of GYKI unless ConA is used. Currently we cannot exclude either interpretation of the effect of ConA upon the antagonism by GYKI of GluR6R receptors.

From our results we propose that there is an allosteric modulatory site on the AMPA receptor which we prefer to call the 'cyclothiazide site', via which cyclothiazide affects the kinetics and extent of antagonism by both competitive and non-competitive antagonists. This putative cyclothiazide site could be important in pharmacological strategies involving antagonism of AMPA/kainate receptors, and may be an important functional element modulating glutamatergic synaptic activity. We re-emphasize that we believe there is another putative modulatory site on the AMPA receptor, appropriately designated a 2,3-benzodiazepine recognition site (Zorumski *et al.*, 1993), through which non-competitive antagonists like GYKI exert their effects. Our current hypothesis is that these two modulatory sites are distinct, yet interact with each other. Our results indicate that reduction of desensitization by cyclothiazide and high affinity blockade by GYKI (reduced and slower in cyclothiazide) characterize the native hippocampal and the recombinant GluRA-D_{flip} AMPA receptors; while low affinity blockade by GYKI and desensitization (both unaffected by cyclothiazide) characterize native DRG and recombinant GluR6R kainate receptors. The importance of the flip/flop region to the action of cyclothiazide upon AMPA receptors has been well demonstrated (Partin *et al.*, 1994; 1995), but it is not known if GYKI antagonism is influenced by alternative splicing or mutations in the flip/flop region, which would imply that the site of action of GYKI is also near the flip/flop region. If true, photoaffinity labelling of these two putative modulatory sites may be required to more precisely establish their similar or distinct identities.

Note added in proof

One binding study (Kessler *et al.*, 1996) and two studies using native and mutated recombinant AMPA and kainate receptors (Johansen *et al.*, 1995; Partin & Mayer, 1996) have also concluded that cyclothiazide and GYKI antagonists interact allosterically.

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References

BETTLER, B., BOULTER, J., HERMANS-BORGMEYER, I., O'SHEA-GREENFIELD, A., DENERIS, E.S., MOLL, C., BORGMEYER, U., HOLLMANN, M. & HEINEMANN, S. (1990). Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. *Neuron*, **5**, 583–595.

BOULTER, J., HOLLMANN, M., O'SHEA-GREENFIELD, A., HARTLEY, M., DENERIS, E., MARON, C. & HEINEMANN, S.(1990). Molecular cloning and functional expression of glutamate receptor subunit genes. *Science*, **249**, 1033–1037.

DONEVAN, S.D. & ROGAWSKI, M.A. (1993). GYKI 52466, A 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/Kainate receptor responses. *Neuron*, **10**, 51–59.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch*, **391**, 85–100.

HERB, A., BURNASHEV, N., WERNER, P., SAKMANN, B., WISDEN, W. & SEEBURG, P.H. (1992). The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron*, **8**, 775–785.

HOLLMANN, M., O'SHEA-GREENFIELD, A., ROGERS, S.W. & HEINEMANN, S. (1989). Cloning by functional expression of a member of the glutamate receptor family. *Nature*, **342**, 643–648.

HUETTNER, J.E. (1990). Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by con A. *Neuron*, **5**, 255–266.

HUETTNER, J.E. & BAUGHMAN, R.W. (1986). Primary culture of identified neurons from the visual cortex of postnatal rats. *J. Neurosci.*, **6**, 3044–3060.

HUETTNER, J.E. & WILDING, T.J. (1995). Differential antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-preferring and kainate-preferring receptors by 2,3-benzodiazepines. *Mol. Pharmacol.*, **47**, 582–587.

JOHANSEN, T.H., CHAUDHARY, A. & VERDOORN, T.A. (1995). Interactions among GYKI-52466, cyclothiazide, and aniracetam at recombinant AMPA and Kainate receptors. *Mol. Pharmacol.*, **48**, 946–955.

JONAS, P. & SAKMANN, B. (1992). Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. *J. Physiol.*, **455**, 143–171.

KEINANEN, K., WISDEN, W., SOMMER, B., WERNER, P., HERB, A., VERDOORN, T.A., SAKMANN, B. & SEEBURG, P.H. (1990). A family of AMPA-selective glutamate receptors. *Science*, **249**, 556–560.

KESSLER, M., ARAI, A., QUAN, A. & LYNCH, G. (1996). Effect of cyclothiazide on binding properties of AMPA-type glutamate receptors – lack of competition between cyclothiazide and GYKI 52466. *Mol. Pharmacol.*, **49**, 123–131.

LIVSEY, C.T., COSTA, E. & VICINI, S. (1993). Glutamate-activated currents in outside-out patches from spiny versus aspiny hilar neurons of rat hippocampal slices. *J. Neurosci.*, **13**, 5324–5333.

LOMELI, H., MOSBACHER, J., MELCHER, T., HOGER, T., GEIGER, J.R.P., KUNER, T., MONYER, H., HIGUCHI, M., BACH, A. & SEEBURG, P.H. (1994). Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science*, **266**, 1709–1713.

MOUDY, A.M., YAMADA, K.A. & ROTHMAN, S.M. (1994). Rapid desensitization determines the pharmacology of glutamate neurotoxicity. *Neuropharmacology*, **33**, 953–962.

NELLGARD, B. & WIELOCH, T. (1992). Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. *J. Cerebral Blood Flow Metab.*, **12**, 2–11.

PARTIN, K.M. & MAYER, M.L. (1996). Negative allosteric modulation of wild-type and mutant AMPA receptors by GYKI 53655. *Mol. Pharmacol.*, **49**, 142–148.

PARTIN, K.M., BOWIE, D. & MAYER, M.L. (1995). Structural determinants of allosteric regulation in alternatively spliced AMPA receptors. *Neuron*, **14**, 833–843.

PARTIN, K.M., PATNEAU, D.K. & MAYER, M.L. (1994). Cyclothiazide differentially modulates desensitization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor splice variants. *Mol. Pharmacol.*, **46**, 129–138.

PARTIN, K.M., PATNEAU, D.K., WINTERS, C.A., MAYER, M.L. & BUONANNO, A. (1993). Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavilin A. *Neuron*, **11**, 1069–1082.

PATERNAIN, A.V., MORALES, M. & LERMA, J. (1995). Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron*, **14**, 185–189.

PATNEAU, D.K., VYKLICKY, L.J. & MAYER, M.L. (1993). Hippocampal neurons exhibit cyclothiazide-sensitive rapidly desensitizing responses to kainate. *J. Neurosci.*, **13**, 3496–3509.

RAMAN, I.M. & TRUSSELL, L.O. (1992). The kinetics of the response to glutamate and kainate in neurons of the avian cochlear nucleus. *Neuron*, **9**, 173–186.

SHARP, R.L., MAY, P.C., MAYNE, N.G., SNYDER, Y.M. & BURNETT, J.P. (1994). Cyclothiazide potentiates agonist responses at human AMPA/kainate receptors expressed in oocytes. *Eur. J. Pharmacol.*, **266**, R1–R2.

SHEARDOWN, M.J., NIELSEN, E.O., HANSEN, A.J., JACOBSEN, P. & HONORE, T. (1990). 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. *Science*, **247**, 571–574.

SOMMER, B., KEINANEN, K., VERDOORN, T.A., WISDEN, W., BURNASHEV, N., HERB, A., KOHLER, M., TAKAGI, T., SAKMANN, B. & SEEBURG, P.H. (1990). Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science*, **249**, 1580–1585.

STERN-BACH, Y., BETTLER, B., HARTLEY, M., SHEPPARD, P.O., O'HARA, P.J. & HEINEMANN, S. (1994). Agonist selectivity is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron*, **13**, 1345–1357.

TANG, C.M., DICHTER, M. & MORAD, M. (1989). Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. *Science*, **243**, 1474–1477.

TARNAWA, I., FARKAS, S., BERZSENYI, P., PATAKI, A. & ANDRASI, F. (1989). Electrophysiological studies with a 2,3-benzodiazepine muscle relaxant: GYKI 52466. *Eur. J. Pharmacol.*, **167**, 193–199.

TRUSSELL, L.O., THIO, L.L., ZORUMSKI, C.F. & FISCHBACH, G.D. (1988). Rapid desensitization of glutamate receptors in vertebrate central neurons. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4562–4566.

WONG, L.A. & MAYER, M.L. (1993). Differential modulation by cyclothiazide and concanavalin A of desensitization at native alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- and kainate-preferring glutamate receptors. *Mol. Pharmacol.*, **44**, 504–510.

YAMADA, K.A. & TANG, C.-M. (1993). Benzothiadiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. *J. Neurosci.*, **13**, 3904–3915.

ZIVKOVIC, I., THOMPSON, D.M., BERTOLINO, M., UZUNOV, D., DIBELLA, M., COSTA, E. & GUIDOTTI, A. (1995). 7-Chloro-3-methyl-3-4-dihydro-2H-1,2,4 benzothiadiazine S,S-dioxide (IDRA 21): a benzothiadiazine derivative that enhances cognition by attenuating DL-alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) receptor desensitization. *J. Pharmacol. Exp. Ther.*, **272**, 300–309.

ZORUMSKI, C.F., YAMADA, K.A., PRICE, M.T. & OLNEY, J.W. (1993). A benzodiazepine recognition site associated with the non-nmda glutamate receptor. *Neuron*, **10**, 61–67.

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