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Cyclothiazide Differentially Modulates Desensitization of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor Splice Variants

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SUMMARY

Agonist responses for flip splice variants of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits GluR-A, -C, and -D are more strongly potentiated by cyclothiazide than are those for the flop forms. Cyclothiazide shows both greater efficacy and higher apparent affinity for potentiation of GluR-A_{flip} versus GluR-A_{flop}. Consistent with higher affinity for the flip splice variant, recovery from potentiation by cyclothiazide proceeds 30 times more slowly for GluR-A_{flip} than for GluR-A_{flop}. In the presence of 300 μM cyclothiazide a 6-fold leftward shift in the kainate dose-response curve for GluR-A_{flip} but not GluR-A_{flop} additionally contributes to a difference in potentiation for these splice variants. Although control responses to glutamate show strong desensitization for both splice variants of GluR-A, in the presence of 100 μM cyclothiazide desensitization is strongly attenuated for GluR-A_{flip}, whereas for GluR-A_{flop} desensitization remains pronounced but with a rate of onset slowed 50-fold, compared with control. In heteromeric AMPA receptors formed from GluR-A and GluR-B, the flip splice variants are dominant for controlling both recovery from potentiation of responses to kainate and block of desensitization of responses to glutamate. Our results suggest that the flip/flop module could directly contribute to the binding site for cyclothiazide, raising the possibility that this site is located in an extracellular receptor domain.

Glutamate receptors generated by expression of the subunits GluR-A, -B, -C, and -D (1), also called GluR1, -2, -3, and -4, respectively (2, 3), form AMPA-preferring receptors, the functional properties of which vary with subunit composition. The most prominent example of subunit-dependent regulation occurs for heteromeric AMPA receptors that contain the GluR-B subunit, in which a glutamine to arginine substitution in the second putative transmembrane domain controls both rectification (4–8) and Ca^{2+} permeability (5–7). Other functional properties that are affected by the subunit composition of heteromeric AMPA receptors include desensitization kinetics and the relative affinity of agonists and antagonists (5, 9, 10). Each of the four AMPA receptor subunits exists as two splice variants, named flip and flop, generated by alternative splicing of two 115-base pair exons that encode 38 amino acids located just proximal to a putative fourth transmembrane domain (10). The functional properties conferred by the flip/flop region have not yet been well characterized but are believed to include effects on both desensitization and the relative efficacy for activation by glutamate and kainate (10).

Recent studies report that both native and recombinant AMPA receptors show allosteric regulation by benzothiadiazines and some structurally related drugs (11–15). Cyclothiazide is the most potent of these compounds identified to date (14). In studies on native AMPA receptors in hippocampal and brainstem auditory neurons, cyclothiazide produced essentially complete block of glutamate-evoked desensitization and marked potentiation of responses to kainate in all cells studied (12, 13, 16). However, in experiments on hippocampal neurons in other laboratories these effects were less consistent (14, 17), and in some cells responses to kainate were resistant to potentiation by cyclothiazide (14). Recent work on neurons of the hilar region of the hippocampus clearly demonstrates differences in sensitivity to cyclothiazide for SMC versus AHI (17). Although the subunit composition of AMPA receptors in hilar neurons is not yet known, this raises the possibility that variability in the expression of AMPA receptor subunits or their splice variants regulates sensitivity to cyclothiazide.

In preliminary experiments on recombinant glutamate receptors, which established the selectivity of cyclothiazide for mod-

ABBREVIATIONS: GluR, glutamate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SMC, spiny mossy cells; AHI, aspiny hilar interneurons; CMV, cytomegalovirus; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEK, human embryonic kidney.

ulation of AMPA but not kainate receptors, we noted that potentiation of responses to kainate and glutamate was stronger for the flip versus flop forms of GluR-A and GluR-B (11). Here we describe splice-dependent effects of cyclothiazide on the activation and desensitization of recombinant AMPA receptors expressed in *Xenopus* oocytes and HEK 293 cells. Our results show that cyclothiazide binds with higher apparent affinity to AMPA receptors containing the flip versus flop splice variant and that recovery from potentiation by cyclothiazide occurs faster for flop versus flip splice variants. In heteromeric AMPA receptors formed by coexpression of both flip- and flop-containing subunits, glutamate-evoked desensitization was strongly attenuated by cyclothiazide, suggesting that the flip splice variant is dominant. Our results raise the possibility that the flip/flop region contributes to the binding site for cyclothiazide. This would be inconsistent with AMPA receptor membrane topology models containing four transmembrane domains (1, 18), because these place the flip/flop module in an intracellular loop between the third and fourth transmembrane regions.

Materials and Methods

cDNA plasmids. For brevity we refer to the flip and flop forms of AMPA receptors using the nomenclature of Keinänen *et al.* (1) and the subscripts i and o, respectively (e.g., GluR-A_i and GluR-A_o). cDNA clones of GluR-A_i, -A_o, -B_i, -B_o, -C_i, -C_o, -D_i, and -D_o in CMV expression vectors were gifts from Dr. P. Seeburg (Center for Molecular Biology, Heidelberg University, Heidelberg, Germany); GluR1 and GluR3 (both flop forms) in pBluescript were gifts from Dr. S. Heinemann (Salk Institute, La Jolla, CA). All plasmids were purified by CsCl gradients. The GluR-A_o(Q582R) mutant was constructed from GluR1 by *dut-ung*-oligonucleotide missense mutagenesis (Bio-Rad) and was subcloned into the CMV expression clone by a *Bgl*II to *Bgl*II fragment swap. A repaired CMV GluR-C_o was made by ligating the GluR3 *Sal*I to *Bgl*II fragment into the CMV GluR-C_i vector that had been prepared by cutting with *Sal*I and *Bgl*II.

***Xenopus* oocyte injections.** Oocytes were surgically obtained from adult oocyte-positive females (Nasco) that were anesthetized by immersion in 0.3% Tricaine (Sigma) for 20 min and then placed on an ice bed. Harvested ovarian lobes were incubated with 1.5 mg/ml collagenase B (Boehringer Mannheim) in calcium-free OR-2 buffer for about 2 hr at room temperature, on an orbital shaker. After thorough washing with Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, pH 7.6], selected stage V-VI eggs were stored at 18° and injected 16–24 hr later. Most experiments were done using DNA injections into the nucleus, which entailed preparing the oocytes by gentle centrifugation (300 × *g*, 10 min) in a plastic grid glued to a Petri dish, to slightly extrude the nucleus. DNA stocks were diluted 1:1 with 400 mM NaCl, 20 mM HEPES, pH 7.5, to a final concentration of 1–2 μg/μl, before injection. In experiments involving expression of heteromeric receptors, injections were made at ratios of 1:2 or 1:5 for GluR-A_iB_i, 1:10 for GluR-A_oB_i and GluR-A_iB_o, 1:5 for GluR-A_iA_o(Q582R), and 1:2 or 1:5 for GluR-A_oB_o. DNA was injected using micropipettes pulled to a diameter of <10 μm. Earlier experiments were done with a Pico-Injector, which injected 5–30 nl/oocyte. Later experiments were done with a Drummond Nanoject, which was set to inject 27.5 nl of DNA/oocyte. Some early experiments were done with RNA injections, essentially as described previously (11), for which 50 nl of *in vitro* transcribed RNA were injected into the oocyte cytoplasm.

Oocyte electrophysiology. Experiments on oocytes were performed under two-electrode voltage clamp (Axoclamp 2A) at a holding potential of −60 mV, in a continuously perfused chamber of approximately 5-μl volume; the flow rate was 250 μl/min, such that the decay time constant of responses to termination of the application of 30 μM kainate was 1.5 ± 0.1 sec. The extracellular solution contained modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ba(NO_3)_2$, 0.41 mM $BaCl_2$, 0.82 mM $MgCl_2$, 15 mM HEPES, pH 7.6], to which was added kainate or glutamate (Sigma), cyclothiazide (gift of Eli Lilly Co.; 20 mM stock solution dissolved in DMSO), or concanavalin A (Sigma). Electrodes of 0.1–3-MΩ resistance were filled with 1 M CsCl and 5 mM EGTA. Current responses were filtered at 20 Hz (Frequency Devices 902) and recorded on a chart recorder (Gould) or a digital data recorder (VR-10B; Instrutech) or acquired by a MacIntosh IIfx computer with an Instrutech ITC-16 interface under control of the program Synapse (Synergistic Research Systems; available by anonymous FTP from Zippy.NIMH.NIH.GOV in the directory \pub\synapse). I-V ramps obtained from −125 mV to +50 mV in the absence of agonist were subtracted from responses obtained in the presence of agonist to estimate rectification of AMPA receptor responses, as described below in greater detail for experiments on HEK 293 cells.

Transfection and rapid perfusion of HEK 293 cells. The HEK 293 cell line (American Type Culture Collection CRL 1573) was grown in minimum essential medium with Earle's salts, 2 mM glutamine, and 10% fetal bovine serum, at 37° in a 5% CO_2 humidified incubator. Approximately 24 hr after plating at low density on fibronectin- or polylysine-coated coverslips, the cells were transfected, according to the method of Chen and Okayama (19), with cDNA for GluR-A_i or -A_o for expression of homomeric receptors or with cDNA for both GluR-A and GluR-B at an A:B ratio of 1:4 for expression of heteromeric receptors containing GluR-A_iB_i, GluR-A_iB_o, or GluR-A_oB_o or at a ratio of 1:2 for GluR-A_oB_i. Whole-cell voltage-clamp recordings from isolated cells were obtained 40–72 hr after transfection, using an Axopatch 1D amplifier (Axon Instruments) and the pClamp 5.5 suite of programs. Extracellular recording solution contained 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 0.01 mg/ml phenol red, pH 7.3 (osmolarity, 305 mOsM). Thick-wall borosilicate glass pipettes with resistances of 2–5 mΩ contained 135 mM CsCl, 10 mM CsF, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM Cs_4BAPTA, pH 7.2 (osmolarity, 305 mOsM). Cyclothiazide was dissolved in DMSO at 20 mM before dilution to 100 μM with extracellular solution; an equivalent final concentration of DMSO (0.5%) was added to all glutamate, kainate, and control solutions not containing cyclothiazide. Concentration-jump experiments were performed using a stepper motor-based rapid perfusion system (20). Salts, biochemicals, and excitatory amino acids were purchased from Aldrich, Diagnostic Chemicals, Molecular Probes, and Sigma.

For co-transfection with GluR-A and GluR-B, hetero-oligomerization in individual cells was verified in two ways, to exclude the presence of a significant proportion of homomeric receptors assembled from GluR-A. A worst-case estimate for expression of homomeric GluR-A versus heteromeric receptors, based on published data of Verdoorn *et al.* (4) for rectification of responses to kainate, was made assuming rectification ratios at +60/−60 mV of 1.9 for GluR-A_iB_i and 0.2 for GluR-A_i, with 5-fold and 20-fold potentiation by cyclothiazide, respectively (Table 1). A rectification ratio of 0.95, the cut-off value used in our experiments, would arise if 24% of the current at −60 mV was due to expression of homomeric GluR-A_i. In the majority of our experiments, rectification ratios were more typically 1.3–1.4, which would reflect only a 10% contribution of homomeric GluR-A. Furthermore, because cyclothiazide actually reduces outward rectification of kainate responses for native AMPA receptors in hippocampal neurons (12) and for recombinant receptors generated from GluR-AB,[1] the expression of homomeric GluR-A is likely to be even lower. In cells for which I-V data were not obtained, we have found that adequate hetero-oligomerization could be confirmed by comparing the relative amplitude of peak responses to glutamate and kainate, because this is significantly smaller for heteromeric receptors generated from GluR-A and GluR-B than for homomeric GluR-A. For GluR-A_i and GluR-A_o the glutamate/kainate

[1] D.K. Patneau and M.L. Mayer, unpublished observations.

ratio was 116 ± 56 and 56 ± 26 (mean ± standard deviation, $n = 6$ each), respectively, whereas the ratio for cells expressing GluR-A in combination with GluR-B, for which I-V plots independently verified hetero-oligomerization, was 8.2 ± 4.6 ($n = 13$). Data from six cells without I-V data, for which the glutamate peak/kainate peak ratio was <12, were included in the results presented here.

Data analysis. Not all oocytes gave a measurable control response in the absence of modulators, due to variability in efficiency of expression of different GluR cDNAs and variability in expression between different batches of oocytes. Therefore, for oocytes that had control responses too small to accurately measure, a value of 1 nA (the lower limit of resolution in our system) was used to calculate potentiation ratios for cyclothiazide and concanavalin A for the experiments illustrated in Fig. 1. This normalization was done for five of 13 oocytes expressing GluR-A_i, three of 10 oocytes expressing GluR-C_i, and three of 11 oocytes expressing GluR-D_i; no data from oocytes expressing the flop isoforms were normalized. Analysis of the GluR-A_i data set excluding the normalized oocyte responses revealed 11.61-fold potentiation of the kainate response by cyclothiazide, compared with 11.57-fold potentiation for the data set that included normalized responses. We therefore concluded that the normalization did not affect the outcome of the analysis.

Dose-response analysis was performed using close to maximally effective concentrations of either cyclothiazide or kainate as appropriate (see Figs. 2 and 3). Responses were fit to the equation $I = I_{max} \times (1/(1 + (EC_{50}/[ligand])^n))$, where I_{max} is the response at a saturating concentration of ligand, EC_{50} is the concentration of ligand producing a half-maximal response, and n is the Hill coefficient. For kainate dose-response curves, to allow correction for rundown, bracketing responses to a given concentration of kainate were recorded at the beginning and end of the control dose-response run and then the dose-response curve for cyclothiazide plus kainate was collected, also with bracketing responses. A function describing the time course of rundown was fit to the bracketing responses for each oocyte, and then the experimental responses were normalized to an interpolated control value. Data values are presented as mean ± standard error. Statistical significance was tested using analysis of variance with *post hoc t* tests.

Results

Cyclothiazide potentiation of responses to kainate is greater for flip than for flop splice variants. The flip/flop variants of AMPA receptor subunits arise from alternatively spliced exons, differing in five amino acid residues conserved for GluR-A through GluR-D (Fig. 1A). In oocytes expressing either the flip or flop variants of homomeric AMPA receptors generated from GluR-A, -C, or -D, responses to 100 μM kainate were consistently potentiated 7–12-fold in the presence of 100 μM cyclothiazide for the flip forms but only 2–4-fold for the flop forms (Fig. 1B). For pooled data from 69 oocytes, there was on average 10.7 ± 1.2-fold potentiation for the flip splice variants but only 3.5 ± 0.4-fold potentiation for the flop splice variants of GluR-A, -C, and -D. Responses for homomeric GluR-B were not examined due to poor functional expression of homomers generated by this subunit (3, 8, 21), but as previously noted (11) potentiation by cyclothiazide was greater for heteromers assembled from GluR-A_iB_i (12.6 ± 1.2-fold) versus GluR-A_oB_o (4.1 ± 0.2-fold). Although the effects of cyclothiazide would be expected to occur at least in part via a reduction in desensitization (11–14, 16), responses to concanavalin A, another drug that reduces desensitization at AMPA receptors (22), did not show consistent differences between the flip and flop forms of GluR-A, GluR-C, or GluR-D (Fig. 1C). Whether this is due to the fact that cyclothiazide and concanavalin A act at different sites or reflects the much weaker action

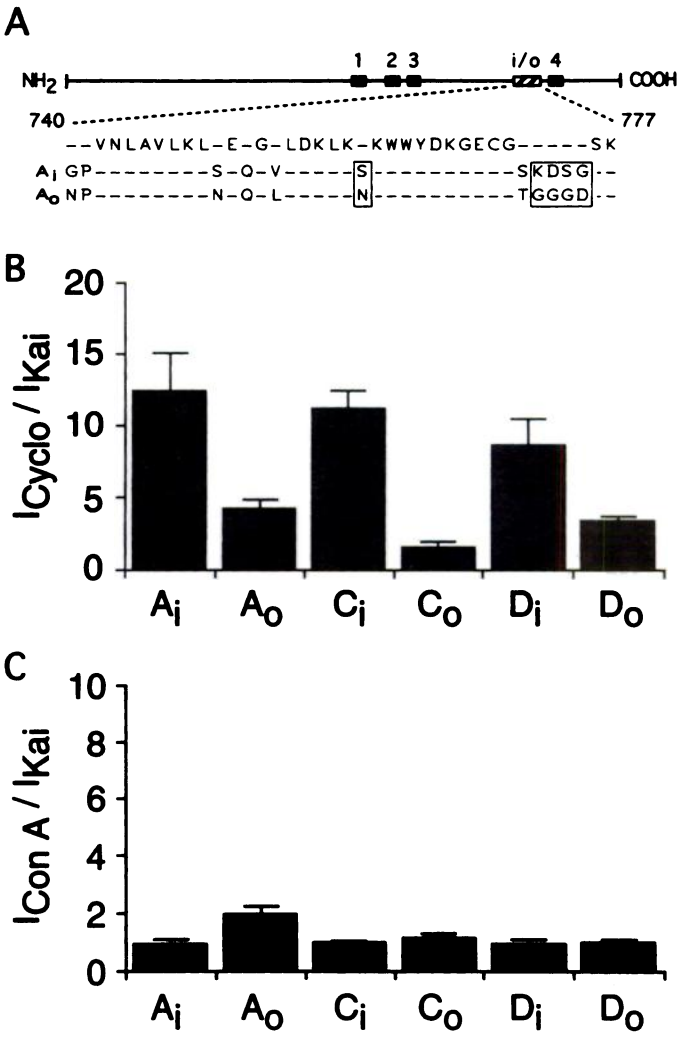

Fig. 1. Cyclothiazide strongly potentiates kainate responses for flip but not flop AMPA receptor splice variants. A, Amino acid sequence comparison at the flip/flop module. *Solid line*, entire coding region for GluR-A; *solid black boxes*, four putative transmembrane regions (1); *hatched box*, flip/flop module (10). Below this is the consensus sequence of the flip/flop module for GluR-A, -B, -C, and -D, which in GluR-A occurs from amino acid residues 740 to 777. In the sequences for GluR-A_i and GluR-A_o *dashes* represent conserved amino acids present in all AMPA receptor subunits, the sequence for which is shown above. *Boxed residues*, "core" (1+4) amino acids that are conserved for all flip or flop variants of GluR-A through GluR-D. B, Potentiation in *Xenopus* oocytes of responses to 100 μM kainate in the presence of 100 μM cyclothiazide. The graph compares the ratio of potentiated responses to control responses for flip and flop isoforms of GluR-A, -C, and -D; for all subunits, the flip splice variant was more strongly potentiated by cyclothiazide. C, Evidence that concanavalin A (0.3 mg/ml) does not differentially potentiate responses to kainate for flip versus flop splice variants. Oocytes expressing homomeric GluR-A_i ($n = 13$ and 8), GluR-A_o ($n = 19$ and 15), GluR-C_i ($n = 10$ and 9), GluR-C_o ($n = 5$ and 4), GluR-D_i ($n = 11$ and 6), and GluR-D_o ($n = 11$ and 10, respectively) were tested for potentiation by cyclothiazide and concanavalin A. *Error bars*, standard error.

of lectins on desensitization at AMPA receptors (11) is not yet known.

The different sensitivity to potentiation by cyclothiazide of kainate responses for the flip and flop splice variants of AMPA receptors could potentially occur because of differences in the affinity or efficacy of cyclothiazide for these splice forms. Alternatively, the amount of desensitization evoked by kainate could be greater for the flip versus flop splice variants, with the

consequence that relief of desensitization by cyclothiazide would produce greater potentiation for the flip splice variants. Because the action of cyclothiazide appeared to be similar for GluR-A, -B, -C, and -D, in experiments designed to characterize further the action of cyclothiazide we used homomeric receptors generated from the flip and flop forms of GluR-A. Dose-response analysis for cyclothiazide revealed both a higher apparent affinity and a greater efficacy for potentiation of responses to kainate for the flip versus flop forms of GluR-A (Fig. 2). For oocytes expressing GluR-A_i the EC_{50} for cyclothiazide was 6.8 ± 0.4 μM (n = 14), with a predicted maximum potentiation of 8.8 ± 1.2-fold. For GluR-A_o, the EC_{50} for cyclothiazide was 62.2 ± 7.9 μM (n = 6), with a predicted maximum potentiation of 6.3 ± 0.9-fold. Dose-response analyses of mean data for oocytes expressing GluR-A_i and GluR-A_o are compared in Fig. 2.

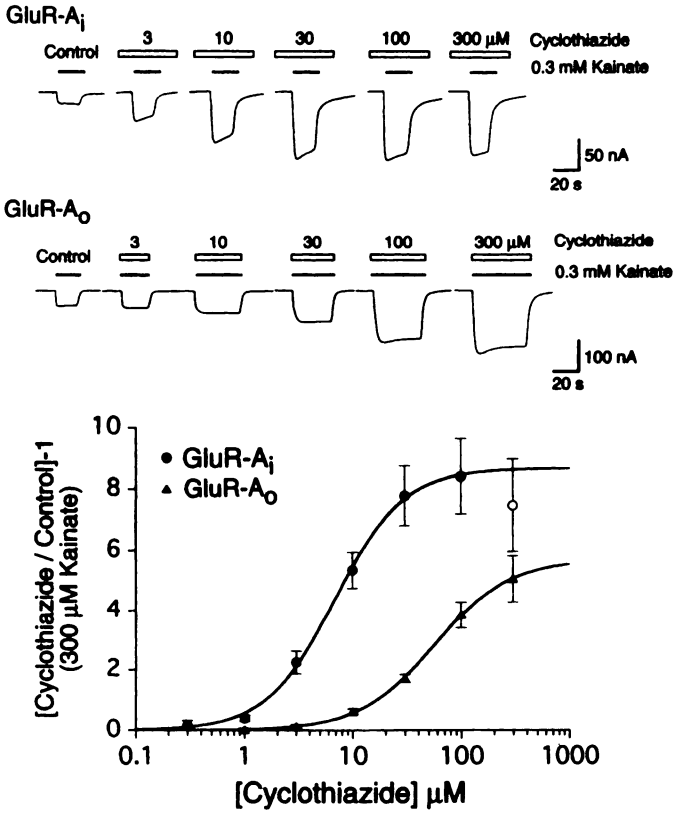

Fig. 2. Cyclothiazide exhibits greater apparent affinity and efficacy for potentiation of kainate responses for GluR-A_i than for GluR-A_o. *Upper*, inward currents from *Xenopus* oocytes evoked by 300 μM kainate (*solid line*), in the presence of increasing concentrations of cyclothiazide (*open bars*), for GluR-A_i and GluR-A_o. Note that 3–10 μM cyclothiazide strongly potentiates responses for GluR-A_i but not GluR-A_o and that, at maximally effective concentrations of cyclothiazide, the response for GluR-A_i shows greater potentiation than does that for GluR-A_o. For GluR-A_i cyclothiazide was applied both before and during application of kainate, because pretreatment with cyclothiazide was required for potentiation to reach equilibrium within the time of application of kainate, whereas for GluR-A_o cyclothiazide was added only during application of kainate. Similar levels of potentiation for GluR-A_o were reached at equilibrium whether or not there was pretreatment with cyclothiazide. *Lower*, dose-response analysis of pooled data (*data points* show mean ± standard error). Responses in the presence of cyclothiazide were normalized to responses to kainate in the absence of cyclothiazide and were fit to the equation in Materials and Methods. EC_{50} values estimated from fits to pooled data were 6.9 μM for GluR-A_i (●) and 56.9 μM for GluR-A_o (▲), with Hill coefficients of 1.38 and 1.26 and maximum potentiation of 8.67 and 5.68, respectively. The data point for GluR-A_i with 300 μM cyclothiazide (○) was excluded from the fit, due to a decrease in potentiation at high concentrations of cyclothiazide.

Inspection of the cyclothiazide dose-response curve for GluR-A_i (Fig. 2) reveals a reduction in potentiation with increases in the concentration of cyclothiazide from 100 to 300 μM, whereas for GluR-A_o potentiation increases over this range. This suggests that at high concentrations cyclothiazide has an additional inhibitory effect on responses to kainate, which is apparent only for GluR-A_i because potentiation for this splice variant reaches a nearly maximum value at 100 μM. For GluR-A_o it is likely that such inhibition would be masked by the increase in potentiation that occurs when the concentration of cyclothiazide is raised from 100 to 300 μM. Due to its poor solubility in physiological solutions, we were unable to demonstrate an inhibitory action of cyclothiazide on responses for GluR-A_o by analysis of dose-response data for concentrations in excess of 300 μM. However, an inhibitory action of cyclothiazide on responses for both GluR-A_i and GluR-A_o was confirmed in subsequent experiments using rapid perfusion techniques (see Fig. 6).

At native AMPA receptors in cultured hippocampal neurons, for which the subunit composition is unknown, 30 μM cyclothiazide produces a 3.0-fold leftward shift in the dose-response curve for kainate (12). To test whether cyclothiazide would produce a similar effect on the affinity of kainate for GluR-A_i and GluR-A_o, we performed a dose-response analysis (Fig. 3). In agreement with previous observations (10), there was no difference in the apparent affinity of kainate for flip and flop splice variants; for GluR-A_i the control EC_{50} for kainate was 73 ± 12 μM (n = 6) and for GluR-A_o the EC_{50} was 60 ± 12 μM (n = 9). However, in the presence of cyclothiazide there was a 6.1-fold increase in apparent affinity for GluR-A_i (EC_{50} of 12 ± 3 μM), with only a small apparent decrease in affinity for GluR-A_o (EC_{50} of 84 ± 6 μM). The leftward shift in the kainate dose-response curve for GluR-A_i resembles that observed for the effects of cyclothiazide on native AMPA receptors (12).

Cyclothiazide blocks desensitization of GluR-A_i but not GluR-A_o. Prior work with both native and recombinant

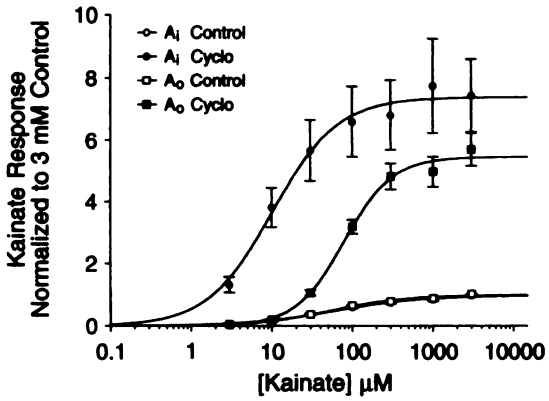

Fig. 3. Cyclothiazide induces a leftward shift of the kainate dose-response curve for GluR-A_i but not for GluR-A_o. Dose-response curves show pooled responses in *Xenopus* oocytes to various concentrations of kainate in the absence or presence of 300 μM cyclothiazide (*data points* show mean ± standard error). Cyclothiazide potentiated the maximum response evoked by kainate for both GluR-A_i and GluR-A_o; however, there was an increase in apparent affinity of kainate only for GluR-A_i. Curve fits to pooled data gave EC_{50} values for control responses of 62 and 56 μM for GluR-A_i and GluR-A_o, respectively; in the presence of 300 μM cyclothiazide the EC_{50} values were 10 and 80 μM, respectively.

AMPA receptors has shown that potentiation of responses to glutamate and kainate by cyclothiazide is at least partially due to a reduction of desensitization (11, 12). Thus, it was surprising that responses to glutamate in oocytes expressing GluR-A_o showed an apparent increase in desensitization in the presence of 100 μM cyclothiazide; this was also true, to a lesser extent, for responses to kainate (Fig. 4). On average, for GluR-A_o the current ratio in the presence of cyclothiazide, measured at the beginning and at the end of responses ("steady state/peak") evoked by agonist applications of 10-sec duration, was 0.17 ± 0.02 (n = 17) for 300 μM glutamate and 0.87 ± 0.03 (n = 16) for 100 μM kainate, whereas for GluR-A_i the ratios were 0.90 ± 0.03 (n = 10) and 1.15 ± 0.06 (n = 13), respectively. Similar differences were observed for GluR-C_o and GluR-D_o versus GluR-C_i and GluR-D_i, with only the flop forms producing strongly desensitizing responses to glutamate in the presence of 100 μM cyclothiazide. The difficulty of accurately measuring the time course and amplitude of desensitization at AMPA receptors expressed in *Xenopus* oocytes prompted us to analyze the effects of cyclothiazide on desensitization using transfected HEK 293 cells and a rapid perfusion system permitting solution exchange on the millisecond time scale.

In the absence of cyclothiazide, responses of HEK 293 cells to 1 mM glutamate were rapidly and strongly desensitizing for both GluR-A_i and GluR-A_o (Fig. 5; Table 1). Responses to 1 mM kainate were much smaller in amplitude and apparently nondesensitizing. In the presence of 100 μM cyclothiazide the peak amplitude of responses to glutamate and kainate was potentiated to a similar extent for both GluR-A_o and GluR-A_i (Fig. 5; Table 1). For GluR-A_i, desensitization was markedly attenuated by cyclothiazide; however, for GluR-A_o the degree of desensitization at equilibrium was similar in the presence and absence of cyclothiazide (Fig. 5; Table 1), although the onset of desensitization in the presence of cyclothiazide was much slower than for control responses (Table 1). As a result, the steady state response to glutamate was potentiated 185-fold for GluR-A_i but only 3-fold for GluR-A_o (Table 1). Responses to 1 mM kainate in the presence of 100 μM cyclothiazide also showed strong desensitization for GluR-A_o (66.5 ± 2.2% desensitization) but not GluR-A_i (5.8 ± 1.3% desensitization). Thus, despite a similar 20-fold potentiation of the peak response to kainate for both splice variants, potentiation of the equilibrium response to kainate was only 7-fold for GluR-A_o versus 20-fold for GluR-A_i (Fig. 5; Table 1).

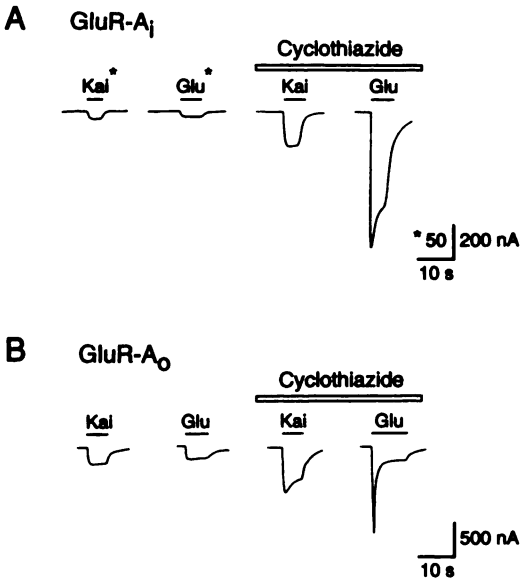

Fig. 4. Glutamate responses in the presence of cyclothiazide show a pronounced time-dependent decay for GluR-A_o but not GluR-A_i. A and B, Inward currents evoked in *Xenopus* oocytes by 100 μM kainate (*Kai*) or 300 μM glutamate (*Glu*) (*solid lines*), in the absence or presence of 100 μM cyclothiazide (*open bar*), for GluR-A_i (A) and GluR-A_o (B). Control responses show little decay; however, in the presence of cyclothiazide the inward current evoked by glutamate shows rapid decay to an equilibrium value for GluR-A_o; this effect is much less pronounced for GluR-A_i. A similar weaker effect is seen for responses to kainate.

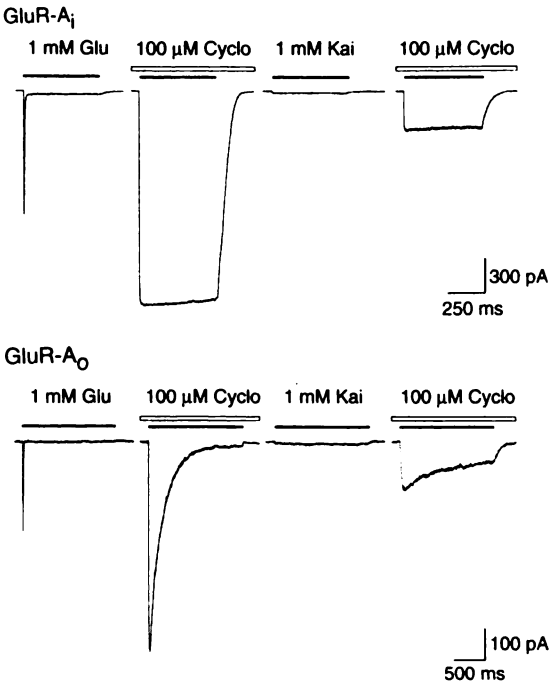

Fig. 5. Concentration-jump experiments on HEK 293 cells reveal block of desensitization by cyclothiazide (*Cyclo*) for GluR-A_i but not GluR-A_o. Control responses to 1 mM glutamate (*Glu*) show rapid and pronounced desensitization for both GluR-A_i and GluR-A_o. In the presence of 100 μM cyclothiazide, the onset of desensitization is markedly slowed for GluR-A_o but the degree of desensitization at equilibrium remains similar in magnitude to control, whereas for GluR-A_i desensitization is essentially blocked. Control responses to kainate (*Kai*) do not show desensitization; however, in the presence of cyclothiazide the kainate response for GluR-A_o shows slow but substantial (57%) desensitization, whereas for GluR-A_i desensitization is minimal (<10%).

Kinetics of recovery from cyclothiazide-evoked potentiation are rapid for GluR-A_o but slow for GluR-A_i. Previous experiments on hippocampal neurons revealed slow on and off kinetics for potentiation by cyclothiazide of re-

sponses to kainate (12). In the present study concentration-jump techniques were used to examine the onset and recovery kinetics for potentiation by cyclothiazide, using both HEK 293 cells and oocytes expressing GluR-A_i or GluR-A_o. When cyclothiazide was applied in the presence of kainate, potentiation developed relatively slowly (Fig. 6). In HEK 293 cells 10–90% rise times for cyclothiazide potentiation were 5.1 ± 0.4 sec for GluR-A_i and 2.8 ± 0.1 sec for GluR-A_o. In *Xenopus* oocytes the 10–90% rise times for GluR-A_i and GluR-A_o were 9.7 ± 0.7 and 9.9 ± 0.6 sec, respectively. For HEK 293 cells expressing GluR-A_i, responses to 1 mM kainate were potentiated 16.6 ± 1.6-fold (n = 5) after application of 100 μM cyclothiazide for 7 sec, whereas for GluR-A_o potentiation was only 8.3 ± 1.5-fold (n = 5); comparable results were obtained in *Xenopus* oocytes for modulation of responses to 300 μM kainate by 15–30-sec applications of 300 μM cyclothiazide (Fig. 6): potentiation was 16.5 ± 3.7-fold (n = 5) and 7.0 ± 1.2-fold (n = 3) for GluR-A_i and GluR-A_o, respectively. For HEK 293 cells transfected with GluR-A_o the degree of potentiation was similar to that observed at equilibrium for kainate responses recorded after pretreatment with cyclothiazide but approximately 2-fold less than the degree of potentiation observed at the peak of the response to kainate (Fig. 5; Table 1).

The kinetics of recovery from potentiation by cyclothiazide showed marked differences for AMPA receptor splice variants and were much slower for GluR-A_i than GluR-A_o (Fig. 6). In HEK 293 cells transfected with GluR-A_i, recovery from potentiation by cyclothiazide occurred too slowly for accurate measurement during continuous application of kainate, and on average potentiation decreased by only 37.4 ± 4.4% at 10 sec after removal of cyclothiazide. In contrast, for GluR-A_o there was complete (100 ± 0.3%) recovery from potentiation by cyclothiazide over the same time period, and the decay of potentiation could be well fit by a single-exponential function with a time constant of 1.3 ± 0.01 sec (n = 5). Similar differences in recovery kinetics were obtained in *Xenopus* oocytes, with much slower recovery from potentiation by cyclothiazide for GluR-A_i than for GluR-A_o (Fig. 6). An advantage of the oocyte preparation was greater tolerance to prolonged application of kainate, such that it was possible to measure the kinetics of recovery from potentiation by cyclothiazide in the continuous presence of kainate for both splice variants of GluR-A (Fig. 6). Such responses were usually better fit by the sum of two exponential components (τ_{fast} and τ_{slow}). For GluR-A_i the time constant for the major component of recovery from potentiation by cyclothiazide (τ_{slow}) was 103 ± 18 sec (relative amplitude, 76 ± 5%), 30 times slower than the time constant for the major component (τ_{fast}) for GluR-A_o, which was 3.7 ± 0.3 sec (relative amplitude, 81 ± 2%).

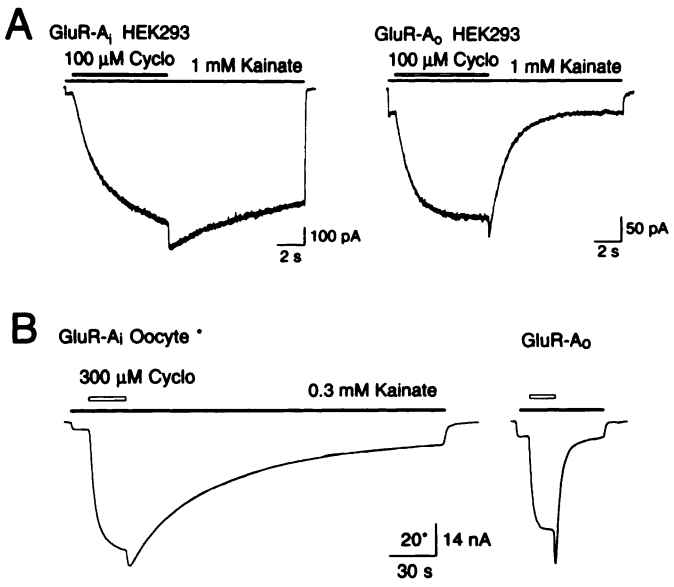

Fig. 6. Recovery from potentiation by cyclothiazide (*Cyclo*) is much slower for GluR-A_i than for GluR-A_o. A, Concentration-jump responses to 100 μM cyclothiazide (*open bar*) applied in the presence of 1 mM kainate (*black bar*), for HEK 293 cells expressing either GluR-A_i (*left*) or GluR-A_o (*right*). After termination of the application of cyclothiazide, there is an increase in amplitude of the response to kainate due to rapid recovery from block by cyclothiazide, followed by a slow decay due to recovery from potentiation; for GluR-A_i the decay of potentiation is too slow to allow complete recovery during the time of application of kainate. B, Similar responses for 300 μM cyclothiazide applied in the presence of 300 μM kainate, recorded from *Xenopus* oocytes expressing either GluR-A_i (*left*) or GluR-A_o (*right*). Note that for GluR-A_i several minutes were required for the decay of potentiation after removal of cyclothiazide. Responses for GluR-A_o and GluR-A_i are shown scaled to the same maximum amplitude in the presence of cyclothiazide; note, however, that potentiation of control responses to kainate was much larger for GluR-A_i than for GluR-A_o.

In both HEK 293 cells and *Xenopus* oocytes there was a rapid increase in amplitude of the cyclothiazide-potentiated response to kainate immediately after removal of cyclothiazide (Fig. 6), followed by a slower decrease in amplitude due to recovery from cyclothiazide-evoked potentiation. We interpret the initial rapid response as being due to recovery from block before the decay of potentiation (12). In HEK 293 cells the 10–90% rise time for recovery from block (93.1 ± 15.2 msec and 45.7 ± 13.5 msec for GluR-A_i and GluR-A_o, respectively) was sufficiently rapid, compared with the decay of potentiation, to permit measurement of the degree of potentiation independently of block. Thus, in the absence of block there was 20.6 ± 1.6-fold potentiation by 100 μM cyclothiazide for GluR-A_i and 9.6 ± 1.8-fold potentiation for GluR-A_o, versus only 16.6 ± 1.6- and 8.3 ± 1.5-fold potentiation, respectively, immediately before removal of cyclothiazide (Fig. 6). The amount of block produced by 100 μM cyclothiazide was 25.2 ± 3.0% and 15.5 ± 1.2% for GluR-A_i and GluR-A_o, respectively, indicating that for both splice variants dose-response analysis of the potentiating effect of cyclothiazide shows distortion at high concentrations of modulator (e.g., Fig. 2).

The action of cyclothiazide on heteromeric AMPA receptors. To determine the relative contributions of the flip/flop module to the properties of heteromeric AMPA receptors, we measured 1) the kinetics of recovery from potentiation by cyclothiazide of responses to kainate using *Xenopus* oocytes and 2) the effects of cyclothiazide on desensitization evoked by glutamate in HEK 293 cells. These experiments were performed using recombinant AMPA receptors generated from GluR-A and GluR-B in which flip subunits were coexpressed with flop subunits. Hetero-oligomerization was independently verified using an established technique, taking advantage of the different rectification properties of GluR-A, which contains a glutamine residue in the second putative transmembrane domain, versus GluR-B, which contains an arginine residue (4). Current-voltage relationships for homomeric GluR-A are characteristically doubly rectifying, but when GluR-A is coexpressed with GluR-B the resulting hetero-oligomers exhibit nearly lin-

TABLE 1

Effects of cyclothiazide on desensitization of homomeric and heteromeric AMPA receptors

Peak and steady state (SS) responses to fast perfusion of 1 mM glutamate and 1 mM kainate were obtained in the presence (cyclo) and absence (control) of 100 μM cyclothiazide, using HEK 293 cells transfected with the indicated subunits, as shown in Figs. 5 and 8. Percent desensitization was calculated as 100 × [(peak − steady state)/peak]. The onset of desensitization for control responses to glutamate, and for GluR-A_o and GluR-A_oB_o for responses to glutamate and kainate in the presence of cyclothiazide, was fit with a single-exponential function (τ_{des}).

	GluR-A_i (n = 6)	GluR-A_iB_i (n = 4)	GluR-A_iB_o (n = 6)	GluR-A_oB_i (n = 4)	GluR-A_oB_o (n = 5)	GluR-A_o (n = 6)
Glutamate responses						
Control desensitization (%)	98.6 ± 0.3	91.7 ± 1.8	97.8 ± 0.3	96.2 ± 1.0	98.4 ± 0.4	99.3 ± 0.3
Cyclo desensitization (%)	12.1 ± 3.6	10.4 ± 3.1	16.8 ± 2.3	8.7 ± 4.1	93.5 ± 1.5	98.3 ± 1.3
Control τ_{des} (msec)	4.1 ± 0.4	10.7 ± 0.4	4.2 ± 0.3	8.1 ± 0.3	3.4 ± 0.3	4.8 ± 0.4
Cyclo τ_{des} (msec)	—[a]	—	—	—	367 ± 55	231 ± 39
Cyclo peak/control peak	3.5 ± 0.4	2.0 ± 0.2	3.8 ± 0.1	2.1 ± 0.1	2.6 ± 0.3	2.4 ± 0.3
Cyclo SS/control SS	185 ± 27	25.4 ± 6.4	155 ± 24	59.5 ± 12.7	12.1 ± 2.4	3.4 ± 0.6
Kainate responses						
Cyclo desensitization (%)	5.8 ± 1.3	<5	<5	<5	34.0 ± 3.0	66.5 ± 2.2
Cyclo τ_{des} (msec)	—	—	—	—	522 ± 52	578 ± 42
Cyclo peak/control peak	21.0 ± 1.7	3.9 ± 0.3	6.1 ± 0.5	4.3 ± 0.4	4.8 ± 0.8	21.4 ± 2.8
Cyclo SS/control SS	19.8 ± 1.7	3.8 ± 0.3	5.9 ± 0.5	4.3 ± 0.3	3.2 ± 0.4	7.1 ± 1.1
Glutamate/kainate						
Control SS ratio	2.0 ± 0.3	0.66 ± 0.11	0.15 ± 0.02	0.37 ± 0.11	0.09 ± 0.01	0.34 ± 0.13
Cyclo SS ratio	19.6 ± 5.1	3.8 ± 0.4	3.7 ± 0.4	4.4 ± 0.4	0.32 ± 0.04	0.20 ± 0.11

[a] —, Weakly desensitizing response.

ear I-V curves (4–8). Although GluR-B expressed alone also generates homomeric receptors with linear I-V curves, in *Xenopus* oocytes functional expression of GluR-B is poor (3, 8, 21) and the amplitude of responses generated by homomeric GluR-B is typically <1% of the response generated by coexpression of GluR-A with GluR-B (21); similar results were obtained in the present experiments. To further minimize the formation of homomeric receptors from GluR-A, we injected 2–10 times more DNA for GluR-B than for GluR-A. Because heteromeric receptors formed by coexpression of GluR-A and GluR-B have functional properties that vary with subunit composition (10, 21), probably due to sequence differences for GluR-A and GluR-B at residues other than the glutamine/arginine site in the second transmembrane domain, a more stringent test of the contribution of the flip/flop module was performed with the mutant GluR-A_o(Q582R) expressed in combination with wild-type GluR-A_i, again using a change in rectification as an assay of hetero-oligomerization.

The kinetics of recovery from potentiation by 100 or 300 μM cyclothiazide of responses to 300 μM kainate were measured as illustrated in Fig. 6B for oocytes expressing GluR-A_i, GluR-A_iB_i, GluR-A_oB_i, GluR-A_iB_o, GluR-A_iA_o(Q582R), GluR-A_oB_o, and GluR-A_o (Fig. 7). Recovery from potentiation was slow (time constant for the major component, >30 sec) for all subunit combinations except GluR-A_oB_o (τ_{off}, 3.9 ± 0.5 sec) and GluR-A_o (τ_{off}, 3.9 ± 0.3 sec), demonstrating that in heteromeric AMPA receptors the flip splice variant dominates the recovery kinetics for cyclothiazide potentiation. For the combination GluR-A_iB_o (1:10), which should be the most stringent test for dominance of the flip module, the recovery kinetics were on average 8.4 times slower than those for the pure flop heteromer GluR-A_oB_o (Fig. 7). Faster recovery kinetics for homomeric GluR-A_i versus GluR-A_iB_i, as well as for GluR-A_iB_o versus GluR-A_iA_o(Q582R), suggest that in heteromeric receptors the flip/flop modules of the GluR-A and GluR-B subunits may not act in an identical manner.

We next turned to experiments on HEK 293 cells to evaluate the relative contributions of the flip/flop module to the block of desensitization by cyclothiazide in heteromeric AMPA receptors. The effects of 100 μM cyclothiazide on desensitization

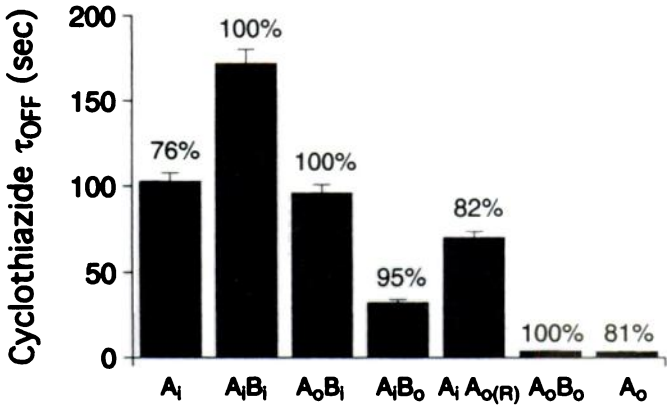

Fig. 7. In heteromeric AMPA receptors the flip splice variant is dominant for slow recovery from cyclothiazide-evoked potentiation. The graph shows time constants (mean ± standard error) for recovery from potentiation by cyclothiazide for homomeric GluR-A_i (n = 5), GluR-A_iB_i (n = 8), GluR-A_oB_i (n = 5), GluR-A_iB_o (n = 5), GluR-A_iA_o(Q582R) (n = 7), GluR-A_oB_o (n = 7), and homomeric GluR-A_o (n = 5). *Numbers above the plots*, relative amplitude of the predominant time constant when recovery was fit by the sum of two exponentials; in all cases the relative amplitude of one component was 3–4 times greater than that of the minor component (values of 100% indicate that recovery was well fit by a single-exponential function). For each cell I-V relationships were determined in the presence of kainate and cyclothiazide and data were included for further analysis only if the rectification ratio, estimated using slope conductance measurements (G_{s+40}/G_{s-60} mV), was >0.9.

evoked by 1 mM glutamate in HEK 293 cells expressing GluR-A_iB_i, GluR-A_oB_i, GluR-A_iB_o, or GluR-A_oB_o are illustrated in Fig. 8 and summarized in Table 1. Cyclothiazide almost fully blocked desensitization of responses to glutamate for all heteromeric receptor combinations containing a flip subunit, similar to the effect observed for homomeric GluR-A_i. The dominance of the flip module was most striking for the combination GluR-A_iB_o (1:4). In contrast, desensitization for heteromeric receptors formed by expression of GluR-A_o with GluR-B_o was only weakly attenuated by cyclothiazide, and with a 2-sec application of glutamate the combination GluR-A_oB_o showed 93.5% desensitization in the presence of cyclothiazide versus 98.4% for control. Similar to the effect observed for GluR-A_o, desensitization for GluR-A_oB_o was slowed approximately 100-

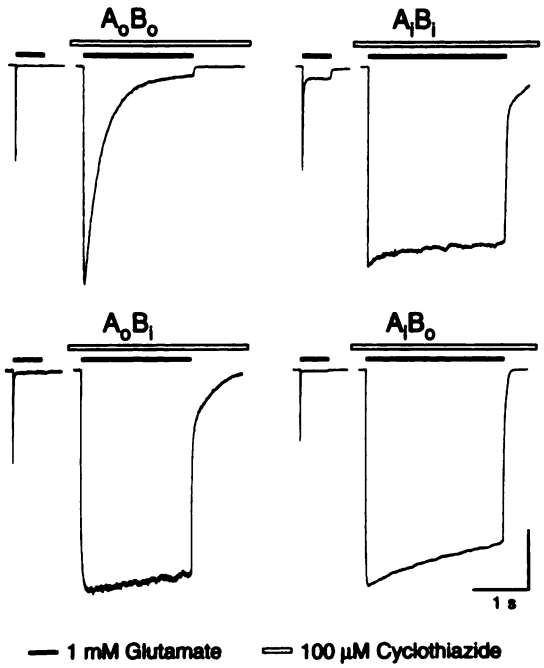

Fig. 8. In heteromeric AMPA receptors block of desensitization by cyclothiazide requires expression of at least one subunit containing the flip splice variant. *Traces*, responses to 1 mM glutamate (*black bar*) for HEK 293 cells transfected with GluR-A_oB_o (1:4), GluR-A_iB_i (1:4), GluR-A_oB_i (1:2), and GluR-A_iB_o (1:4), recorded in the presence (*open bar*) and absence of 100 μM cyclothiazide. In the presence of cyclothiazide desensitization is substantially reduced for all subunit combinations except GluR-A_oB_o; note that desensitization of the control response for GluR-A_iB_i was less than for other subunit combinations. Rectification ratios measured from current responses at +60/−60 mV for steady state control responses to kainate (for GluR-A_oB_o) or responses to kainate or glutamate in the presence of cyclothiazide (for other subunits) were >0.95. *Scale bar*, 300 pA for GluR-A_oB_o, 600 pA for GluR-A_iB_i, 240 pA for GluR-A_oB_i, and 800 pA for GluR-A_iB_o.

fold in the presence of cyclothiazide (Fig. 8; Table 1). In cells expressing GluR-A_oB_o the responses to kainate also exhibited strong desensitization, whereas the responses to kainate for all other receptor combinations were essentially nondesensitizing (Table 1). Thus, data from both oocytes and HEK 293 cells indicate that the flip splice variant is dominant in determining whether cyclothiazide can block desensitization evoked by glutamate and kainate at heteromeric AMPA receptors, independently of whether the flip-containing subunit is either GluR-A or GluR-B.

Our experiments extend previous work by Sommer *et al.* (10), suggesting that desensitization of responses to glutamate for heteromeric receptors assembled from GluR-A and GluR-B is affected by both subunit and splice variant composition, but they differ from that study in that we find strong desensitization for GluR-A_oB_o and GluR-A_oB_i, combinations for which desensitization in response to glutamate was previously not well resolved (10). For responses to 1 mM glutamate, we found that the time constant for the onset of desensitization for heteromeric receptors containing the GluR-B flip subunit, i.e., GluR-A_iB_i and GluR-A_oB_i, was significantly slower ($p < 0.01$) than that for homomeric GluR-A_i or GluR-A_o or for heteromeric receptors containing the GluR-B flop subunit, i.e., GluR-A_iB_o and GluR-A_oB_o (see Table 1). Thus, it appears that the kinetics of AMPA receptor desensitization are in part controlled by hetero-oligomerization with the GluR-B subunit, with GluR-B_i conferring slower kinetics than GluR-B_o. Although responses to glutamate were strongly desensitizing both for homomeric GluR-A and for heteromeric receptors formed by GluR-A and GluR-B, responses to glutamate in cells expressing GluR-A_iB_i exhibited significantly ($p < 0.01$) less desensitization (91.7%) than did the other subunit compositions examined (all ≥96% desensitization). This difference in the magnitude of desensitization for control responses to glutamate has the consequence that for GluR-A_iB_i potentiation by cyclothiazide measured at steady state (25-fold) is reduced, compared with GluR-A_i (185-fold) and GluR-A_iB_o (155-fold).

In the absence of cyclothiazide the relative efficacy of glutamate versus kainate for equilibrium responses at heteromeric AMPA receptors varied with subunit composition, as described previously (10). As shown in Table 1, the efficacy of glutamate relative to kainate was lower for GluR-A_oB_o (0.09) and GluR-A_iB_o (0.15) than for GluR-A_iB_i (0.66) and GluR-A_oB_i (0.37); hence, the GluR-B flip splice variant appears to be dominant in raising the efficacy of kainate. In the presence of cyclothiazide, there were no subunit-dependent differences in relative efficacy for kainate versus glutamate between GluR-A_iB_i, GluR-A_iB_o, and GluR-A_oB_i, and in all cases kainate appeared to act as a partial agonist, exhibiting approximately 25% efficacy relative to glutamate. These results suggest that, for control responses at recombinant AMPA receptors, differences in the relative efficacy of kainate and glutamate measured at equilibrium reflect, at least in part, variation in desensitization with subunit composition.

Discussion

Site of interaction of cyclothiazide with AMPA receptor splice variants. We show here that the flip/flop splice variants of AMPA receptors are differentially modulated by cyclothiazide. However, the site at which cyclothiazide acts is not yet clear. Cyclothiazide could bind directly to AMPA receptor subunits either intra- or extracellularly, with accessory or regulatory proteins that are associated with the receptor complex, or within the lipid bilayer of the plasma membrane. In previous experiments we showed that intracellular dialysis of hippocampal neurons with 100 μM cyclothiazide does not reduce desensitization evoked by glutamate or interfere with the action of cyclothiazide applied in the extracellular solution (12). It therefore appears that the site of action is neither intracellular nor in the plasma membrane. The fact that cyclothiazide potentiates the activity of recombinant AMPA receptors expressed in HEK 293 cells and *Xenopus* oocytes to a similar extent as that observed for native AMPA receptors expressed on neurons (12–14, 16), glia (24), and the NT2-N cell line (25) argues against an interaction with a regulatory protein or second messenger system that modulates the receptor complex. Therefore, we consider it most likely that cyclothiazide binds to a specific site on the extracellular domain of AMPA receptor complexes. The differential effects of cyclothiazide on the flip versus flop splice variants could occur either because amino acids encoded by this region contribute directly to the binding site for cyclothiazide or because these residues trigger conformational changes that affect the binding of cyclothiazide at a different site.

Putative extracellular location of the flip/flop region. If amino acids in the flip/flop module interact directly with cyclothiazide, then these residues would need to be located in

the extracellular domain of AMPA receptors. The membrane topology of GluR ion channels has not yet been resolved; however, several models based on protein hydropathy plots have been suggested. The first models for the topology of AMPA and *N*-methyl-D-aspartate receptor subunits proposed four transmembrane regions (1, 8, 18, 23), similar to other ligand-gated ion channels (27); this gives an intracellular location for the flip/flop region. Models with five transmembrane regions have also been proposed for AMPA and *N*-methyl-D-aspartate receptors (26, 28, 29) and predict an extracellular location for the flip/flop region. Although our results with cyclothiazide are provocative, definitive evidence concerning the transmembrane topology of GluR subunits will require additional approaches, such as insertion of immunogenic epitopes into putative extracellular and intracellular loops or the further identification of amino acid residues that are either glycosylated or phosphorylated. Photoaffinity labeling of the cyclothiazide binding site, with agents confined to either the extracellular or intracellular side of the membrane, might also provide information on receptor topology.

Mechanism of action of cyclothiazide. This study further characterizes the differential modulation by cyclothiazide of the flip versus flop splice variants of AMPA receptors, but our experiments were not designed to address the mechanism of action of cyclothiazide. The multiple effects of cyclothiazide on native AMPA receptors, which include a reduction in desensitization (12, 14, 16, 17), a slowing of deactivation after the removal of agonist (12), and a decrease in the rate of decay of AMPA receptor-mediated excitatory synaptic currents (14), are most likely indicative of either multiple binding sites for cyclothiazide or multiple mechanisms of action. Cyclothiazide potentiation of responses to kainate probably reflects both reduction of desensitization, due to either a change in the kinetics for onset or recovery from desensitization or steric hindrance of the conformational change underlying receptor desensitization, and a slowing of the rate constant for channel closing, thereby affecting both deactivation kinetics and apparent affinity for agonists.

We propose, as one possible mechanism of action of cyclothiazide, that AMPA receptors that have bound cyclothiazide cannot desensitize until after cyclothiazide dissociates and that the kinetics of dissociation of cyclothiazide, which are much slower for the flip versus flop splice variants of GluR-A, contribute to differential modulation of desensitization. This hypothesis is supported by the greater apparent affinity of cyclothiazide for potentiation of kainate responses for GluR-A_i versus GluR-A_o (Fig. 2) and, in the presence of cyclothiazide, strong desensitization for GluR-A_o but with a reduced rate of onset, whereas for GluR-A_i there was essentially complete block of desensitization evoked by 500-msec glutamate applications (Fig. 5). An alternative mechanism that could account for differential modulation of desensitization for GluR-A_i versus GluR-A_o would be a reduction in the rate of onset of desensitization for cyclothiazide-bound receptors, with much greater slowing for GluR-A_i than for GluR-A_o. Because hetero-oligomers assembled from the flip and flop splice variants of GluR-A and GluR-B differ in their desensitization properties (Table 1 and Ref. 10), further dissection of the mechanisms underlying the differential modulation by cyclothiazide is likely to be inherently complex. In the future, experiments designed to address the differential modulation by cyclothiazide of flip versus flop splice variants and the mechanism of action of cyclothiazide on AMPA receptors are likely to converge on common themes that eventually may elucidate the molecular mechanisms underlying AMPA receptor desensitization.

Subunit composition of native AMPA receptors. Our experiments suggest that benzothiadiazines could potentially be used as functional tools to distinguish between native AMPA receptors containing flip versus flop subunits. The results of such experiments would be easy to interpret if there exist subtypes of native AMPA receptors that incorporate only a single splice variant. It is not yet clear whether this occurs *in vivo*, but recent single-cell polymerase chain reaction analysis of AMPA receptor subunit mRNA expression in hippocampal neurons grown in dissociated culture suggests selective expression of flop isoforms in a subpopulation of type II neurons (30). The dominance of the flip splice variant in regulating sensitivity to cyclothiazide suggests that it may not be possible to distinguish between receptors containing only flip splice variants and AMPA receptor heteromers containing both splice variants, unless there are effects of flip/flop subunit stoichiometry on the modulatory action of cyclothiazide. Additional experiments are required to address this, and in particular it remains to be determined whether the effect of cyclothiazide becomes weaker as the ratio of flop to flip subunits increases. This is of interest because *in situ* hybridization analysis of AMPA receptor subunit mRNA reveals selective expression of flip splice variants early in development, with a delayed expression of flop splice variants that is coincident with the peak period of synaptic development throughout the forebrain (31).

Experiments on the hilar region of the hippocampus reveal differences in sensitivity to cyclothiazide (17), which potentially could arise as a result of differential expression of flip versus flop AMPA receptor splice variants in SMC versus AHI. The apparent affinity of cyclothiazide is greater in SMC versus AHI and desensitization of glutamate responses in SMC is essentially abolished in the presence of 100 μM cyclothiazide, whereas responses to glutamate in AHI still exhibit significant desensitization. However, the kinetics of onset of glutamate-evoked desensitization in the presence of 100 μM cyclothiazide in AHI are apparently much faster than shown here for GluR-A_o or GluR-A_oB_o. Because we have not yet examined the effect of cyclothiazide on desensitization kinetics for all possible combinations of the flip and flop splice variants of GluR-A–GluR-D, it is not yet possible to predict the extent to which apparent differences in the effect of cyclothiazide on AHI versus SMC reflect the stoichiometry of splice variants or the expression of different combinations of AMPA receptor subunits.

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