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Structural Determinants of Allosteric Regulation in Alternatively Spliced AMPA Receptors

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Summary

The flip and flop splice variants of AMPA receptors show strikingly different sensitivity to allosteric regulation by cyclothiazide; heteromers assembled from GluR-A and GluR-B also exhibit splice variant-dependent differences in efficacy for activation by glutamate and kainate. The sensitivity for attenuation of desensitization by cyclothiazide for homomeric GluR-A was solely dependent upon exchange of Ser-750 (flip) and Asn-750 (flop), and was unaffected by mutagenesis of other divergent residues. In contrast, substantial alteration of the relative efficacy of glutamate versus kainate required mutation of multiple residues in the flip/flop region. Modulation by cyclothiazide was abolished by mutation of Ser-750 to Gln, the residue found at the homologous site in kainate-preferring subunits, whereas introduction of Ser at this site in GluR6 imparted sensitivity to cyclothiazide.

Introduction

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, which mediate fast excitatory responses at the majority of CNS synapses (Trussell et al., 1994; Collingridge and Lester, 1989), show considerable heterogeneity in their functional properties (Livsey and Vicini, 1992; Hestrin, 1993; McBain and Dingledine, 1993; Jonas et al., 1994; Raman et al., 1994), most likely as a result of alternative splicing (Sommer et al., 1990; Gallo et al., 1992; Köhler et al., 1994), RNA editing (Sommer et al., 1991; Lomeli et al., 1994), and assembly of heteromers from various members of the AMPA receptor gene family (Boulter et al., 1990; Keinänen et al., 1990; Mosbacher et al., 1994). Studies on the alternatively spliced forms of AMPA receptor subunits named flip and flop, which are generated by alternative usage of two exons each encoding 38 amino acids (Sommer et al., 1990), have revealed marked differences in sensitivity to allosteric regulation of desensitization by cyclothiazide (Partin et al., 1994). Whether the differential regulation by cyclothiazide is due to an interaction between the drug and receptor at amino acids encoded by the flip/flop module, or whether these residues direct a conformational change that affects drug interaction at a distal site, is not known. The discovery that drugs which reduce AMPA receptor desensitization also modulate fast excitatory synaptic transmission (Isaacson and Nicoll, 1991; Vyklicky et al., 1991; Yamada and Rothman, 1992; Yamada and Tang, 1993) and enhance performance in animal models of learning (Staubli et al., 1994; Nicoletti et al., 1992) provides further stimulus for understanding the molecular mechanisms underlying the differential sensitivity of AMPA receptor splice variants to allosteric regulation by cyclothiazide.

To address this issue, we have performed site-directed mutagenesis, exchanging amino acids in each of the three regions that differ between the flip and flop splice variants of AMPA receptors and expressing the mutant receptors either in Xenopus laevis oocytes or transiently transfected 293 cells, using as an assay each of the multiple changes in AMPA receptor activity that occur owing to allosteric modulation by cyclothiazide (Partin et al., 1994). In addition, we analyzed the same mutations for their effect on the relative efficacy of glutamate and kainate as agonists. This is of interest because it is thought that for $A_{flop}B_{flop}$ the lower amplitude of steady state responses to glutamate compared with kainate reflects stronger desensitization for this subunit combination than for $A_{flip}B_{flip}$ (Sommer et al., 1990; Mosbacher et al., 1994; Partin et al., 1994; Lomeli et al., 1994). Our results show that the molecular determinant critical for allosteric regulation by cyclothiazide is necessary but insufficient to regulate the efficacy of glutamate versus kainate without the exchange of additional residues in the flip/flop module.

Results

Mutational Strategy

Alignment of the two 38 amino acid regions encoded by exons for the flip and flop modules (Figure 1) shows three distinct regions of heterogeneity, separated by regions of amino acid identity (Sommer et al., 1990). The first region of heterogeneity is at the N-terminal boundary of the alternatively spliced region and therefore is juxtaposed next to the RNA editing site in GluR-B, GluR-C, and GluR-D (Lomeli et al., 1994): in these subunits the first pair of residues for the flip isoforms is ThrPro, whereas in the flop isoforms the sequence is AsnAla; in GluR-A the corresponding sequences are GlyPro and AsnPro. The second region lies 10 residues away from the Thr/Asn site and consists of a single amino acid difference, Ser for the flip isoforms and Asn for the flop isoforms. The third region of heterogeneity lies close to the C-terminal boundary of the alternatively spliced region and consists of 4 consecutive residues, LysAspSerGly for flip isoforms and GlyGlyGlyAsp for flop.

Our mutational strategy to define the amino acids that are critical for allosteric modulation by cyclothiazide was to make single amino acid substitutions, converting flip (i) residues to flop (o) residues and vice versa. Point mutations are referred to using the conventional single letter nomenclature, e.g., A_iS750N and A_oN750S (Figure 1). Because our initial experiments with point mutations in the C-terminal boundary did not influence modulation by cyclothiazide, we then constructed mutations that con-

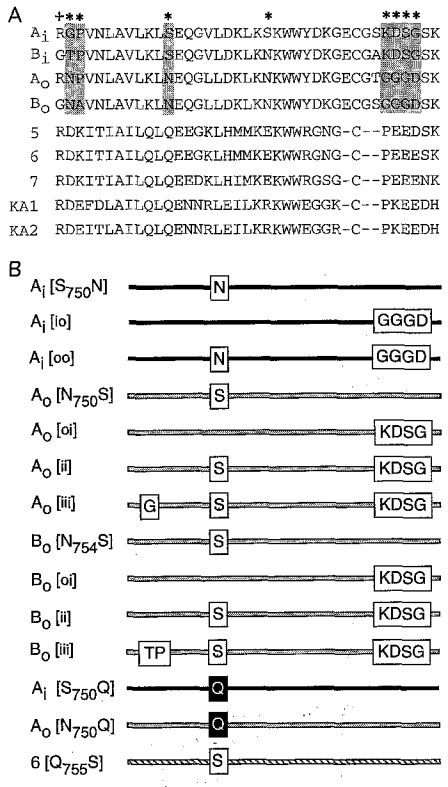

Figure 1. Sequence Alignment and Mutants of the Flip/Flop Domain and Homologous Region in Kainate Receptors

(A) Alignments for the flip and flop versions of GluR-A (residues 739–777 of the mature polypeptide) and GluR-B (residues 743–781), and for the kainate receptor subunits GluR5–7, KA1, and KA2. Asterisks above the alignment indicate positions at which mutations were introduced; a plus sign indicates an RNA editing site. Shaded residues indicate three regions of heterogeneity between AMPA receptor splice variants: TP-S-KDSG for flip and NA-N-GGGD for flop, except that for GluR-A the first pair of conserved amino acids differs from those for other subunits. For kainate receptor subunits, there is a glutamine (Q755 in GluR6) at the position corresponding to the serine/asparagine site in AMPA receptors.

(B) Schematic of the flip/flop module for the mutants constructed for the present experiments, with each mutant identified by the abbreviations shown on the left; boxes indicate the residues introduced by site-directed mutagenesis and their location in the flip/flop module.

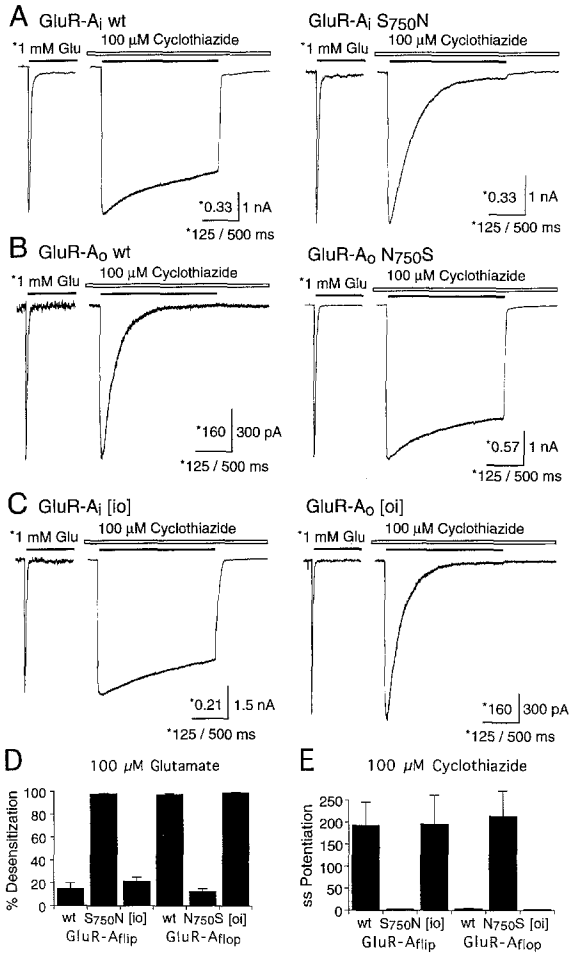

Figure 2. Modulation of Desensitization by Cyclothiazide for the Flip and Flop Versions of Homomeric AMPA Receptors Is Controlled by a Single Amino Acid

(A and B) The left-hand pairs of traces show responses to 1 mM glutamate (closed bars) before and during application of 100 µM cyclothiazide (open bars). The right-hand pairs of traces show comparable responses for mutants in which a single amino acid was exchanged from its flip (serine) to its flop (asparagine) form and vice versa. Control responses to glutamate are shown on a 4-times expanded time scale and are normalized to the peak amplitude of responses recorded in the presence of cyclothiazide.

(C) Responses for mutants in which a set of 4 conserved amino acids was exchanged from the flip (KDSG) to the flop (GGGD) form and vice versa.

(D and E) Summaries of equilibrium desensitization and potentiation of responses to 1 mM glutamate, both recorded in the presence of 100 µM cyclothiazide for 5–7 cells per subunit (bars show mean ± SEM). Experiments were performed using whole-cell recording from 293 cells with agonists applied by concentration jump.

verted all 4 amino acids in this region. These mutations are referred to as A_i[io] or A_o[oi], where the first letter in brackets designates the Ser/Asn site and the second letter refers to the four residues near the C-terminus of the alternatively spliced region. Combined mutations that altered both the Ser/Asn site and the 4 residues are referred to as A_i[oo] and A_o[ii]. The additional mutation in A_o[ii] to the flip form of a single amino acid difference at the N-terminal boundary of the flip/flop module (position 740: asparagine to glycine) is referred to as A_o[iii]. The nomenclature used for comparable GluR-B mutants is similar to that for GluR-A, with the exception that the construction of B_o[iii] from B_o[ii] required the additional exchange of 2 amino acids at positions 744 and 745 from their flop (AsnAla) to their flip (ThrPro) forms. GluR-B_o mutants were assayed in combination with wild-type and mutant GluR-A_o subunits, because homomeric receptors generated from GluR-B express very small currents (Boulter et al., 1990; Herlitze et al., 1993).

Prior studies with wild-type AMPA receptors assembled from GluR-A and GluR-B have revealed a variety of functional differences between flip and flop splice variants, including the extent of attenuation of desensitization of responses to glutamate by cyclothiazide, the potency for potentiation of responses to kainate by cyclothiazide, the kinetics of recovery from potentiation by cyclothiazide,

Table 1. Effects of Cyclothiazide on Homomeric Mutant AMPA Receptors

	WT A_i	A_iS750N	A_i[io]	WT A_o	A_oN750S	A_o[oi]	A_iS750Q	A_oN750Q
Desensitization								
Control (%)	96.8 ± 1.8	97.0 ± 1.3	97.7 ± 2.2	98.4 ± 2.0	97.8 ± 1.5	99.6 ± 0.5	97.5 ± 1.1	98.5 ± 1.5
Cyclothiazide (%)	15.0 ± 12.6	97.8 ± 1.5	21.4 ± 8.1	97.4 ± 2.5	12.5 ± 6.4	99.0 ± 0.8	93.9 ± 4.4	98.2 ± 1.3
Control τ_{des} (ms)	4.1 ± 1.1	4.5 ± 1.2	4.6 ± 1.6	4.9 ± 1.6	3.9 ± 0.8	5.1 ± 2.2	2.7 ± 0.5	3.3 ± 0.6
Cyclothiazide τ_{des} (ms)	[a]	249 ± 44	[a]	214 ± 41	[a]	229 ± 85	4.5 ± 1.0	5.1 ± 1.4
Cyclothiazide Potentiation								
Glutamate steady-state potentiation	157 ± 52	3.2 ± 0.8	196 ± 66	3.8 ± 1.2	214 ± 58	2.4 ± 0.9	1.0 ± 0.1	1.2 ± 0.2
Cyclothiazide EC_{50} (μM)	6.8 ± 0.4[d]	37 ± 9.1	9.5 ± 0.6	62 ± 7.9[d]	7.1 ± 1.2	104 ± 9.6	[b]	[b]
Recovery time (s)	96 ± 7	5.2 ± 0.5	126 ± 6	4.9 ± 1.0	84 ± 7	4.5 ± 0.3	[c]	[c]
Agonist Potency								
Kainate EC_{50} Control (μM)	60 ± 12[d]	38 ± 2.5	38 ± 9.1	47 ± 4.0[d]	24 ± 2.3	37 ± 2.5	42 ± 5	36 ± 3
Kainate EC_{50} Cyclothiazide (μM)	12 ± 3[d]	36 ± 4	6.1 ± 1.8	59 ± 6.9[d]	3.6 ± 0.5	39 ± 8	39 ± 4	34 ± 3

The rate of onset and extent of desensitization of responses to 1 mM glutamate were recorded in the presence and absence of 100 μM cyclothiazide, in 293 cells transfected with various mutants as indicated. τ_{des} was estimated from single exponential fits as shown in Figure 5. Data from the same series of experiments were analyzed to estimate potentiation of equilibrium responses to 1 mM glutamate by 100 μM cyclothiazide, except for GluR-A_iS750Q and GluR-A_oN750Q, which were assayed in Xenopus oocytes using 300 μM glutamate. For all mutants, the EC_{50} for potentiation of responses to 300 μM kainate by cyclothiazide was estimated from dose–response analysis using Xenopus oocytes injected with AMPA receptor cDNAs. The time for 63% recovery from potentiation of responses to 300 μM kainate by 100 μM cyclothiazide was also estimated using Xenopus oocytes. To study agonist potency, responses to kainate were recorded from Xenopus oocytes in the presence and absence of 100 μM cyclothiazide. Values given are mean ± SEM of data from 4–11 oocytes or 293 cells, as appropriate. WT, wild-type.

[a] Desensitization was too slow to estimate τ_{des} accurately.

[b] Potentiaiton was too small to estimate the EC_{50} for cyclothiazide.

[c] Potentiation was too small to estimate a recovery time.

[d] Data from Partin et al., 1994.

the presence or absence of cyclothiazide-evoked shifts in the kainate dose–response curve, and the differences in agonist efficacy as determined by the relative amplitude of equilibrium responses to kainate versus glutamate (Sommer et al., 1990; Partin et al., 1994). Selected mutant AMPA receptors were assayed for each of these parameters, as well as for desensitization of control responses to glutamate applied by rapid perfusion.

A Single Amino Acid Controls Block of Desensitization by Cyclothiazide

Similar to wild-type recombinant AMPA receptors (Mosbacher et al., 1994; Partin et al., 1994), homomeric receptors assembled from the GluR-A mutants generated for this study all showed pronounced desensitization in response to 1 mM glutamate (Figure 2). In 293 cells transfected with mutant AMPA receptors, the rate of onset of desensitization was rapid (time constant typically < 5 ms) and not significantly different for the various mutants examined (Table 1); the extent and kinetics of onset of desensitization were also indistinguishable from results obtained for wild-type GluR-A_i and GluR-A_o (Partin et al., 1994; Mosbacher et al., 1994). In the presence of 100 μM cyclothiazide, desensitization was strikingly reduced for A_oN750S and A_i[io], with results similar to those obtained for wild-type GluR-A_i (Partin et al., 1994). For A_iS750N and A_o[oi], the rate of onset of desensitization was slowed approximately 50-fold in the presence of 100 μM cyclothiazide (time constant typically > 200 ms; Table 1), but at equilibrium the extent of desensitization remained pronounced (Figure 2), similar to the behavior obtained for wild-type GluR-A_o. Such experiments indicate that, for homomeric AMPA receptors assembled from GluR-A_i, the marked attenuation of desensitization by cyclothiazide requires a serine residue at position 750, with no difference in behavior when the C-terminal set of 4 conserved amino acids is in the flip or flop form. Conversely, in GluR-A_o the presence of an asparagine residue at position 750 is sufficient to produce a 50-fold slowing in the rate of onset of desensitization in the presence of cyclothiazide (Table 1), with no effect observed on exchange of the C-terminal set of 4 amino acids to the flip form (Figure 2).

Recovery from Potentiation by Cyclothiazide

A second striking difference in the effect of cyclothiazide on wild-type AMPA receptor flip and flop splice variants was observed upon analysis of the kinetics of recovery from potentiation of responses to kainate; recovery is extremely slow for GluR-A_i but rapid for GluR-A_o, consistent with the higher apparent affinity of cyclothiazide for GluR-A_i (Partin et al., 1994). To examine the influence of amino acids conserved in flip versus flop splice variants on recovery from potentiation, we recorded the effect of brief applications of 100 μM cyclothiazide on responses to 1 mM kainate using Xenopus oocytes expressing mutant AMPA receptors (Figure 3). Recovery from potentiation was slow for A_oN750S and for A_i[io], with close to 100 s typically required for 63% recovery, similar to results obtained for wild-type GluR-A_i (Table 1). For A_iS750N and A_o[oi], the potentiating effect of cyclothiazide was rapidly reversible; 5 s or less was typically required for 63% recovery, similar to results obtained for wild-type GluR-A_o (Table 1). Thus, identical to the requirements for attenuation of desensitization by cyclothiazide, slow recovery from potentiation of responses to kainate also requires a serine residue at position 750, with no influence of the C-terminal set of 4 amino acids. Conversely, introduction of an asparagine residue at position 750 increases the rate of recovery from potentiation by cyclothiazide, irrespective of whether the C-terminal set of 4 conserved amino acids is in the flip (KDSG) or flop (GGGD) version.

Dose-Response Analysis for Responses to Cyclothiazide and to Kainate

For wild-type AMPA receptors, the concentration of cyclothiazide required for half-maximal potentiation of responses to kainate is approximately 9-fold lower for GluR-A_i than for GluR-A_o (Partin et al., 1994). To determine

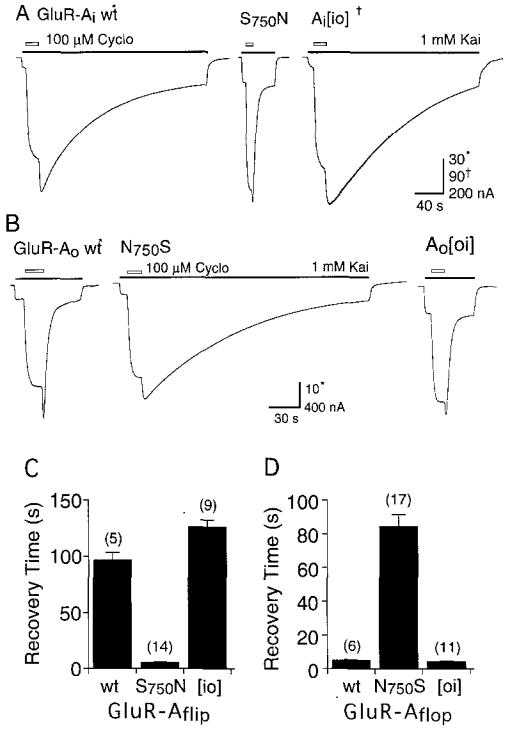

Figure 3. In Homomeric AMPA Receptors, Recovery from Potentiation by Cyclothiazide Is Controlled by the Same Site Regulating Allosteric Modulation of Desensitization

(A and B) Potentiation of responses to 0.3 mM kainate (closed bars) during application of 100 μM cyclothiazide (open bars) for wild-type receptors (left) and for mutants in which either the single residue at position 750 (middle) or the C-terminal set of 4 amino acids (right) was exchanged from the flip to the flop form and vice versa. Following removal of cyclothiazide, there is a transient increase in amplitude of responses to kainate before the onset of the decay of potentiation; this results primarily from rapid recovery from an inhibitory effect of cyclothiazide itself, which does not distinguish between splice variants, and thus is probably not determined by the flip/flop module.

(C and D) Summaries of measurements of the time required for 63% recovery from potentiation by cyclothiazide for wild-type and mutant receptors (bars show mean ± SEM, with the number of oocytes tested indicated above each plot). Experiments were performed using two-electrode voltage-clamp recording from Xenopus oocytes.

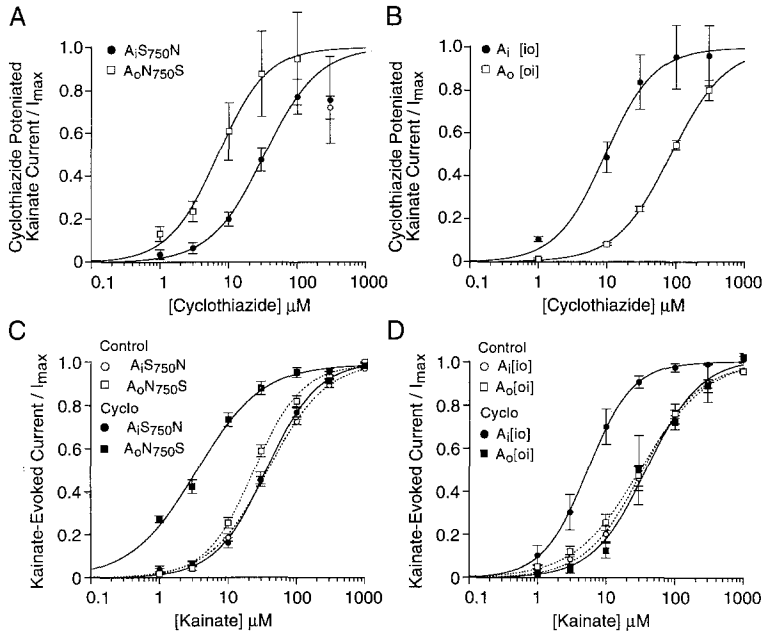

Figure 4. Cyclothiazide and Kainate Dose–Response Analysis for Mutants

Dose–response analysis for the potentiating effects of cyclothiazide in homomeric AMPA receptors reveals regulation by the residue at position 750, but not by the C-terminal residues in the flip/flop module.

(A and B) Dose–response curves for potentiation of responses to 300 μM kainate by cyclothiazide. Similar to the difference observed for wild-type GluR-A_{flip} versus wild-type GluR-A_{flop}, the potency of cyclothiazide is higher for potentiation of responses for A_oN750S and A_i[io] than for A_iS750N and A_o[oi]. Data points show mean ± SEM of observations from 4–8 oocytes per subunit. For the analysis shown in (A), data points at 300 μM cyclothiazide were excluded from the fit due to a reduction in potentiation resulting from inhibition at high concentrations of cyclothiazide; in these experiments, DMSO (1.5%) was present in all solutions.

(C and D) Dose–response curves for control responses to kainate (dashed lines); in the presence of 100 μM cyclothiazide (solid lines), there is a leftward shift for A_oN750S and A_i[io] but not for A_iS750N and A_o[oi].

whether the amino acids that regulate attenuation of desensitization by cyclothiazide also control sensitivity to cyclothiazide, dose–response curves were recorded in Xenopus oocytes expressing mutant AMPA receptors (Figures 4A and 4B). Such experiments revealed that the greater potency of cyclothiazide for potentiation of responses to kainate for GluR-A_i versus GluR-A_o requires the same amino acid residue at position 750 that regulates block of desensitization. Thus, similar to wild-type $GluA_i$, the potency of cyclothiazide was high for A_oN750S and A_i[io] but low for A_iS750N and A_o[oi]. Together, these results reveal no effect of the C-terminal set of 4 conserved amino acids, with the potency of cyclothiazide determined exclusively by the presence of a serine or asparagine residue at position 750 (Table 1).

Analysis of the effects of cyclothiazide on kainate dose–response curves (Figures 4C and 4D) revealed a shift to the left by 100 μM cyclothiazide of 7-fold for A_oN750S and 6-fold for A_i[io], similar to the 5-fold shift seen for wild-type GluR-A_i, with no effect of cyclothiazide on the potency of kainate for A_iS750N and A_o[oi]. Together with previous results for block of desensitization, these experiments reveal a single amino acid switch between the flip and flop versions of GluR-A to be sufficient to determine each component of allosteric modulation by cyclothiazide assayed in the present experiments, most likely indicating a common mechanism of action.

Sensitivity to Cyclothiazide in Homomeric AMPA and Kainate Receptors Regulated by a Glutamine Residue Conserved in Kainate Receptor Subunits

The regulation of sensitivity to cyclothiazide for the flip and flop versions of GluR-A by a single amino acid, and considerable sequence homology between AMPA and kainate receptors in this region (Figure 1), raised the possibility that the lack of sensitivity of kainate receptors to cyclothiazide (Partin et al., 1993; Wong and Mayer, 1993) might reflect sequence differences between AMPA and kainate receptors over this region. Within 11 amino acids surrounding the Ser/Asn site in AMPA receptors, sequence alignments for each of the five known kainate receptor subunits reveal 6–7 residues to be identical or to have highly conservative substitutions. In AMPA receptors 9/11 residues are identical over the same region; however, at the position corresponding to the Ser/Asn site in AMPA receptors, all five kainate receptor subunits express a glutamine residue (Figure 1).

Substitution of a glutamine at position 750 in both A_iS750Q and A_oN750Q essentially abolished sensitivity to cyclothiazide (Figure 5A), without altering agonist potency (Figure 5B). The absence of potentiation of responses to kainate and glutamate by cyclothiazide was particularly striking for the response of A_iS750Q to 300 μM glutamate (100 μM cyclothiazide/control = 0.996 ± 0.082; n = 11), since for wild-type GluR-A_i there was a 157 ± 52–fold potentiation (n = 6). The cyclothiazide-evoked shift in EC_{50} for activation by kainate was also abolished for A_iS750Q (Figure 5B). In both the presence and absence of cyclothiazide, the potency of kainate was similar to control values obtained for wild-type GluR-A_i and for other mutant receptors (Table 1), indicating that although the introduction of Gln-750 is sufficient to abolish sensitivity to cyclothiazide, it does not confer the high sensitivity to kainate typical of wild-type kainate receptors, and that is seen in AMPA receptor chimeras containing kainate receptor sequences thought to encode the agonist-binding site (Stern-Bach et al., 1994). Consistent with these results, when assayed in 293 cells, cyclothiazide failed to block desensitization for both A_iS750Q and A_oN750Q (Figure 5C). The rate of onset of and the extent of desensitization for control responses to 1 mM glutamate in 293 cells transfected with these Gln-750 mutants were similar to values obtained

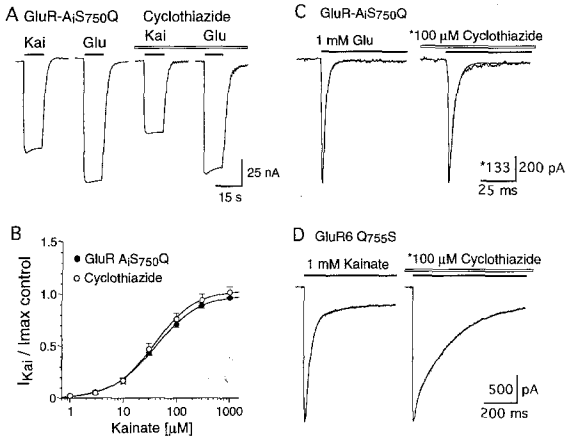

Figure 5. Sensitivity to Cyclothiazide Blocked by a Glutamine Residue in Homomeric AMPA and Kainate Receptors

(A) Responses to 0.3 mM kainate and 0.3 mM glutamate recorded from a Xenopus oocyte expressing A_iS750Q, before and during application of 100 μM cyclothiazide. In contrast to results obtained for wild-type GluR-A_i, cyclothiazide fails to produce a clear potentiation of responses to either agonist; this is particularly striking in the case of glutamate, for which potentiation would normally be greater than 150-fold.

(B) Analysis of kainate dose–response curves recorded from Xenopus oocytes expressing A_iS750Q in the presence (n = 5) and absence (n = 6) of 100 μM cyclothiazide. Data points show mean ± SEM fit with a logistic equation, which gave EC_{50}s for kainate of 40.1 and 38.0 μM before and during application of cyclothiazide. In contrast to results obtained for wild-type GluR-A_i, there was no leftward shift in the dose–response curve in the presence of cyclothiazide.

(C) Responses to 1 mM glutamate (closed bars) before and during application of 100 μM cyclothiazide (open bar) for a 293 cell transfected with A_iS750Q; lines fit to the desensitizing component phase are single exponential functions. In contrast to results obtained for wild-type GluR-A_i, cyclothiazide has no significant effect on the extent (control, 98.4%; cyclothiazide, 97.3%) or rate of onset of desensitization (control τ_{des}, 2.2 ms; cyclothiazide τ_{des}, 3.8 ms); similar results were obtained for A_oN750Q.

(D) Responses to 1 mM kainate (closed bars) before and during application of 100 μM cyclothiazide (open bar) for a 293 cell transfected with GluR6Q755S; lines fit to the desensitizing phase are double exponential functions. In contrast to the complete lack of sensitivity to cyclothiazide observed for wild-type GluR6, for GluR6Q755S there was a clear reduction in the rate of onset of desensitization (63% decay times: control, 25.3 ms; cyclothiazide, 55.5 ms), although for kainate applications 1.5 s in duration, the extent of desensitization was similar in the presence (88.4%) and absence (89.1%) of cyclothiazide.

for both wild-type AMPA receptors and the other mutants constructed for this study, and was unaltered in the presence of cyclothiazide (Table 1). Together, these results indicate that the lack of sensitivity to cyclothiazide was not a result of a loss of desensitization for control responses to glutamate.

The block of AMPA receptor sensitivity to cyclothiazide by introduction of a glutamine residue at position 750, and sequence homology between AMPA and kainate receptors at positions surrounding this site, suggests that the absence of kainate receptor sensitivity to cyclothiazide might arise from the presence of a glutamine residue in kainate receptor subunits. To examine this possibility, we constructed the mutant GluR6Q755S. In contrast to results obtained for the AMPA receptor mutant A_iS750Q, the desensitization kinetics of control responses to 1 mM kainate for GluR6Q755S were not identical to those for wild-type GluR6, with GluR6Q755S showing a reduced rate of onset of desensitization (wild type: τ_{1des} = 9.6 ± 1.0 ms, A_1 = 93.3% ± 1.6%, τ_{2des} = 156 ± 27 ms; GluR6Q755S: τ_{1des} = 29.6 ± 2.4 ms, A_1 = 84.8% ± 1.4%, τ_{2des} = 318 ± 39 ms). In addition, there was less desensitization for GluR6Q755S (90.6% ± 1.6%) than for wild-type GluR6 (97.8% ± 0.6%). However, in contrast to the cyclothiazide-insensitive responses obtained for wild-type GluR6, the rate of onset of desensitization for GluR6Q755S was clearly slowed in the presence of 100 μM cyclothiazide (Figure 5D). For wild-type GluR6, the time required for desensitization to reach 63% of maximum for control responses to 1 mM kainate was 11.6 ± 1.3 ms (n = 13), with no change in the presence of cyclothiazide (11.2 ± 1.2 ms; n = 11). For GluR6Q755S, the values were 25.5 ± 1.5 ms (n = 16) for control responses and 56.8 ± 4.2 ms (n = 9) in the presence of cyclothiazide. However, other functional properties characteristic of GluR6, including a high affinity for kainate, lack of sensitivity to AMPA, and pronounced block of desensitization by concanavalin A (Egebjerg et al., 1991; Partin et al., 1993) were maintained in GluR6Q755S (data not shown).

To control for nonspecific effects resulting from mutations at position 750 in AMPA receptors, we introduced an alanine at position 750; for this mutant, potentiation by cyclothiazide was between those for GluR-A_i and GluR-A_o (data not shown), indicating that substitution with an amino acid other than serine or asparagine is not sufficient to prevent modulation by cyclothiazide. Further evidence for a critical role of residue 750 was obtained by mutating a second serine residue at position 760, which for GluR-A but not other AMPA receptor subunits (Figure 1) is exchanged by alternative splicing from serine (flip) to asparagine (flop); in GluR-B, GluR-C, or GluR-D both splice variants encode an asparagine at the position homologous to S760 in GluR-A_i. The mutation A_iS760N behaved similarly to wild-type GluR-A_i when assayed in Xenopus oocytes for potentiation by cyclothiazide and for the kinetics of recovery from potentiation (data not shown). Thus, the effects on differential regulation by cyclothiazide of serine to asparagine exchange in GluR-A depend critically on the location of the altered site, and are restricted to position 750.

Sensitivity to Cyclothiazide in Heteromeric AMPA Receptors

In heteromeric AMPA receptors assembled from wild-type GluR-A and GluR-B, the flip splice forms are dominant for regulating sensitivity to block of desensitization by cyclothiazide (Partin et al., 1994). To determine whether the single amino acid residue at position 750, which controls sensitivity to cyclothiazide in homomeric receptors assembled from GluR-A, is sufficient to regulate desensitization of responses for heteromeric AMPA receptors, we assayed the sensitivity to cyclothiazide of heteromeric receptors for which an asparagine to serine exchange was made in either the GluR-A_o or GluR-B_o subunits (Figure 6). Cyclothiazide produced pronounced attenuation of desen-

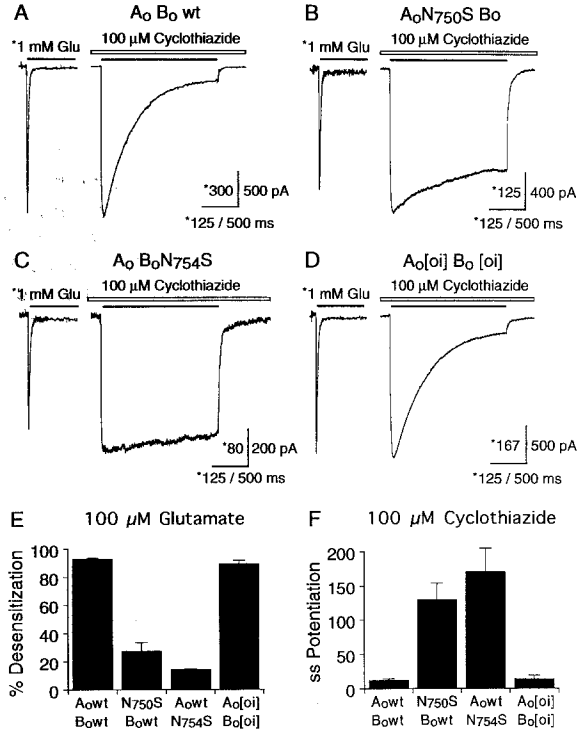

Figure 6. For Heteromeric AMPA Receptors, a Serine Residue at the Ser/Asn Site in either GluR-A or GluR-B Is Sufficient to Confer Strong Sensitivity to Attenuation of Desensitization by Cyclothiazide

For each subunit combination, the left-hand trace shows a control response to 1 mM glutamate (closed bars); traces on the right were recorded during application of 100 μM cyclothiazide (open bars). Control responses to glutamate are shown on a 4-times expanded time scale and are normalized to the peak amplitude of responses recorded in the presence of cyclothiazide.

(A) A 50-fold slowing of the rate of onset of desensitization by cyclothiazide for wild-type A_oB_o, but with pronounced desensitization at equilibrium.

(B and C) Strong attenuation of desensitization by cyclothiazide for the combinations $A_oN750SB_o$ and A_oB_oN754S.

(D) Results for A_o[oi]B_o[oi]. When the C-terminal set of 4 amino acids was exchanged from the flop (GGGD) to the flip (KDSG) version, there was pronounced desensitization in the presence of cyclothiazide, but with a rate of onset slowed 50-fold compared with control, similar to results obtained for wild-type A_oB_o.

(E and F) Summaries of equilibrium desensitization and potentiation of responses to 1 mM glutamate, both recorded in the presence of 100 μM cyclothiazide for 5–7 293 cells per subunit (bars show mean ± SEM).

sitization evoked by glutamate for both $A_oN750SB_o$ (100 μM cyclothiazide; 27.6% ± 6.2% desensitization; n = 7) and A_oB_oN754S (100 μM cyclothiazide; 14.3% ± 0.6% desensitization; n = 5), indicating that in heteromeric receptors a serine residue on either the GluR-A or GluR-B subunit is sufficient to confer high sensitivity to cyclothiazide. Control responses to 1 mM glutamate were not influenced by these mutations, with 95.9% ± 1.0% desensitization, τ_{des} = 5.0 ± 0.19 ms for $A_oN750SB_o$, and 95.9% ± 1.8% desensitization, τ_{des} = 4.9 ± 0.40 ms for A_oB_oN754S. Although not verified experimentally, these results suggest that cyclothiazide would also strongly attenuate desensitization for receptors assembled from GluR-A_o and GluR-B_o subunits both containing an asparagine to serine mutation; in contrast, when the C-terminal set of 4 amino acids was exchanged from the flop (GGGD) to the flip (KDSG) version in both GluR-A and GluR-B subunits, desensitization remained pronounced in the presence of cyclothiazide (Figure 6). Control responses to glutamate for A_o[oi]B_o[oi] showed 96.3% ± 1.1% desensitization, with 89.8% ± 2.3% desensitization (n = 6) in the presence of 100 μM cyclothiazide and a rate of onset slowed 50-fold compared with control (τ_{des} control = 5.0 ± 0.47 ms; τ_{des} cyclothiazide = 367 ± 39 ms), similar to results obtained for the wild-type combination A_oB_o (τ_{des} control = 3.6 ± 0.11 ms; τ_{des} cyclothiazide = 324 ± 12 ms; n = 7).

In Heteromeric AMPA Receptors, Separate Functional Domains Control Sensitivity to Cyclothiazide and Equilibrium Amplitude of Responses to Kainate versus Glutamate

For heteromeric AMPA receptors assembled from various combinations of the flip and flop forms of GluR-A and GluR-B, prior work reveals an 8-fold difference in the amplitude of equilibrium responses to maximally effective concentrations of kainate and glutamate for A_iB_i compared with A_oB_o, with intermediate behavior observed for A_iB_o and A_oB_i (Sommer et al., 1990). Recent work suggests this reflects subunit-dependent differences in the extent of desensitization of responses to glutamate (Mosbacher et al., 1994; Partin et al., 1994). Because the rapidity and extent of desensitization make accurate measurement of the peak amplitude of agonist responses at AMPA receptors technically difficult, the effects of mutations in the flip/flop region were analyzed using the original approach of Keinänen et al. (1990): comparing the amplitude of equilibrium responses to 300 μM kainate with those to 300 μM glutamate. To assay changes in sensitivity to cyclothiazide, in the same experiments we measured the time for 63% recovery from potentiation of responses to kainate by cyclothiazide. The results of these experiments indicate that, compared with the site controlling sensitivity to cyclothiazide, additional residues in the flip/flop modules of heteromeric AMPA receptors determine the relative amplitude of equilibrium responses to kainate versus glutamate (Figure 7). The amplitude ratio (kainate/glutamate) of responses for these agonists was highest for wild-type A_oB_o (27.1 ± 0.7) and decreased approximately 3-fold upon exchange of asparagine to serine at positions 750 and 754 in GluR-A and GluR-B (10.4 ± 4.1), with a similar 3-fold decrease upon exchange from GGGD to KDSG of the C-terminal set of 4 amino acids in both GluR-A and GluR-B (8.7 ± 0.6), but remained below the value for A_iB_i (2.5 ± 0.7). Results presented earlier for the heteromeric combinations A_oB_oN750S, $A_oN750SB_o$, and A_o[oi]B_o[oi] suggest that, since a serine residue in either the GluR-A or GluR-B subunit is sufficient to allow cyclothiazide to attenuate desensitization, a similar effect would occur for $A_oN750SB_oN750S$, A_o[ii]B_o[ii], and A_o[iii]B_o[iii]. The 20-fold slowing of the rate of recovery from potentiation by cyclothiazide observed for $A_oN750SB_oN750S$ contrasts with the lack of effect observed for A_o[oi]B_o[oi], indicating that sepa-

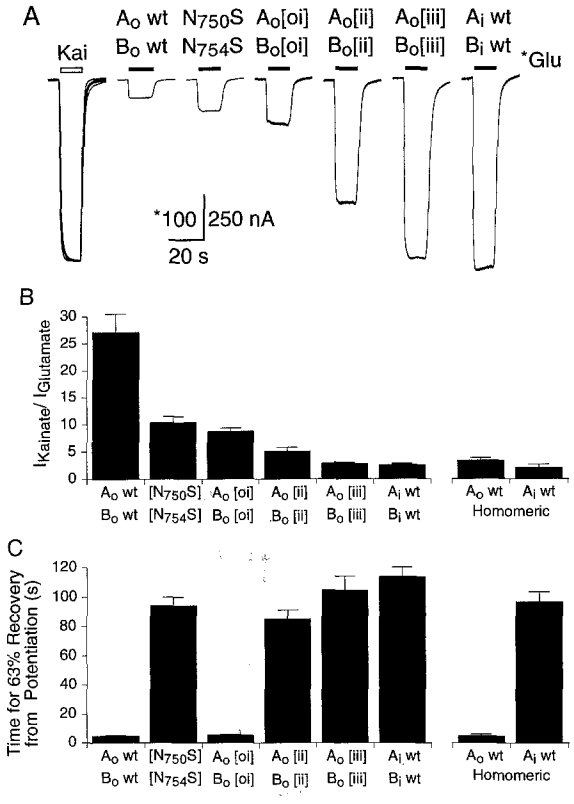

Figure 7. The Relative Amplitude of Equilibrium Responses to Kainate and Glutamate Is Controlled by Amino Acids Additional to the Ser/Asn Site Regulating Sensitivity to Cyclothiazide

(A) Superimposed responses to 300 μM kainate (open bar, traces on left) recorded from 6 oocytes for heteromers generated either from wild-type A_oB_o or A_iB_i or from mutants into which flip splice variant residues were progressively substituted in both the GluR-A and GluR-B subunits, as indicated above the records; traces on the right show individual responses from the same oocytes to 300 μM glutamate. The scale for glutamate responses is 2.5 times that for kainate; to allow comparison between oocytes, responses to kainate are scaled to the same size; the scale bar applies to responses recorded for A_oB_o (scale factors for other subunit combinations ranged from 0.9 to 6.4).

(B) Summary of the subunit dependence of the relative amplitude of responses to 300 μM kainate and 300 μM glutamate recorded from 8–15 oocytes (mean ± SEM) per subunit combination for heteromers, and for comparison from 7 oocytes each for homomeric GluR-A.

(C) Summary of measurements of the time required for 63% recovery from potentiation of responses to 300 μM kainate by 300 μM cyclothiazide for the same subunit combinations.

rate residues control allosteric regulation by cyclothiazide and the relative amplitude of responses to kainate and glutamate (Figure 7B). The times required for 63% recovery from potentiation were 5.6 ± 0.7 s for A_oB_o, 95.9 ± 6.2 s for $A_oN750SB_oN750S$, 5.3 ± 0.5 s for A_o[oi]B_o[oi], and 113.5 ± 6.4 s for A_iB_i.

The combined exchange of both asparagine to serine and the conserved set of C-terminal 4 amino acids in A_o[ii]-B_o[ii] produced a 5.4-fold increase in the relative amplitude of equilibrium responses to glutamate versus kainate. This was significantly ($p < .05$) lower than the 11-fold difference observed between wild-type A_oB_o and A_iB_i (Figure 7A), even though the kinetics of recovery from potentiation by cyclothiazide were similar for these combinations (Figure 7B). The additional exchange of residues at the N-terminal end of the flip/flop modules that differ between splice variants in GluR-A and GluR-B fully restored the behavior of wild-type flip receptors; the relative amplitude of responses to kainate versus glutamate was 2.8 ± 0.2 for A_o[iii]B_o[iii], with 104.4 ± 9.5 s required for 63% recovery from potentiation (these values are not significantly different from results obtained for wild-type A_iB_i). Although it has been shown that AMPA receptor responses evoked by kainate desensitize by approximately 30% (Patneau et al., 1993), responses to glutamate desensitize to a much greater extent (Table 1). Thus, in Figure 7A our superimposition of responses to kainate reflects the assumption that subunit-specific changes in the relative amplitude of responses to glutamate occur because the mutations in the flip/flop module that alter desensitization do so to a greater extent for responses to glutamate than for kainate. Thus, amino acids that regulate recovery from potentiation by cyclothiazide of responses to kainate in heteromers generated from GluR-A and GluR-B are also critical for attenuation of desensitization, but are not sufficient for determining the relative amplitude of control responses to kainate versus glutamate.

Discussion

Recent experiments to determine the transmembrane topology of non-N-methyl-D-aspartate (NMDA) receptors, using insertional mutagenesis of glycosylation sites (Hollmann et al., 1994) or antigenic epitopes (Bennett and Dingledine, 1995), have resulted in topological models for AMPA receptors that place the flip/flop region extracellularly (but see also McGlade-McCulloh et al., 1993). Based on analysis of the agonist binding properties of AMPA and kainate receptor chimeras (Stern-Bach et al., 1994), a recent model for AMPA receptors places the flip/flop region in an extracellular domain labeled S2. Together with amino acids located in the N-terminal S1 domain, residues in S2 are believed to form the agonist-binding site. According to this model, amino acids in the flip/flop module are not in close contact with agonist, consistent with our finding that the mutations generated for the present study did not affect the potency of kainate or the rate of onset of or extent of desensitization of control responses to glutamate (Table 1). The results of our experiments, combined with models based on studies with chimeric receptors, raise the possibility that part of the binding site for cyclothiazide in GluR-A includes residue 750. Hence, although the flip and flop variants of AMPA receptors show differences in amino acid sequence at three separate areas (Figure 1), for homomeric receptors assembled from GluR-A, their strikingly different sensitivity to cyclothiazide can be fully explained by a single amino acid difference at position 750; an identical conclusion can be reached for heteromeric receptors assembled from GluR-A and GluR-B. Although we have not performed mutagenesis experiments for subunits other than GluR-A and GluR-B, we have confirmed that homomeric receptors generated from GluR-C_i and GluR-D_i also show marked attenuation of desensitization by cyclothiazide, identical to that observed for GluR-A_i,

whereas for GluR-C_o and GluR-D_o, desensitization remains pronounced in the presence of cyclothiazide, but with a greatly reduced rate of onset, similar to results obtained for GluR-A_o (data not shown) and further suggesting that results obtained for GluR-A and GluR-B mutants extend to other AMPA receptor subunits. Finally, not only was sensitivity to cyclothiazide abolished in GluR-A by the substitution of Gln for Ser-750, a residue conserved in all cyclothiazide-insensitive kainate receptors, but the substitution of serine for glutamine conferred cyclothiazide sensitivity upon GluR6 (Figure 5). Together, these results provide definitive evidence for a critical role of this site in regulating the selectivity of cyclothiazide. Our data further support the conclusions of Stern-Bach et al. (1994)—that the membrane topologies of AMPA- and kainate-preferring receptors share structural similarity—but do not provide definitive evidence whether the whole of the M3–M4 loop is extracellular, or whether an additional membrane-crossing region exists within this region (Roche et al., 1994; Taverna et al., 1994).

Structural models for glutamate receptors based on homology with lysine/arginine/ornithine-binding protein (LAOBP), for which crystal structures have been solved at atomic resolution for the ligand-bound and ligand-free states (Oh et al., 1993), suggest that Ser-750 will be located in a solvent-accessible α-helical region (O'Hara et al., 1993; Kuryatov et al., 1994; Stern-Bach et al., 1994). Of interest, helical wheel models for the flip/flop module and for the homologous region of LAOBP show similar features and place Ser-750 on one face of an amphipathic helix together with other polar residues, including Arg-739, which in GluR-B, GluR-C, and GluR-D is a site at which RNA editing introduces an arginine to glycine substitution, with effects on the extent of and kinetics of recovery from desensitization (Lomeli et al., 1994). High resolution structural analysis, or photoaffinity labeling experiments, will ultimately be required to confirm that residues surrounding Ser-750 directly contribute to the binding of cyclothiazide to AMPA receptors, since it is not yet possible to exclude that the flip/flop module is involved in conformational transitions underlying desensitization, with binding of cyclothiazide to some other site acting via an allosteric effect.

Our finding that, for heteromeric receptors assembled from GluR-A_o and GluR-B_o, the presence of a serine residue in either the GluR-A or GluR-B subunit confers high sensitivity to cyclothiazide, would be consistent with desensitization resulting from a concerted transition involving all subunits, with the binding of cyclothiazide to even a single subunit blocking the conformational transition underlying desensitization. Consistent with this hypothesis, kinetic analysis of responses to cyclothiazide has revealed that for A_oB_o cyclothiazide does not slow the rate of onset of desensitization per se, but rather, that the slow onset of desensitization in the presence of cyclothiazide results from dissociation of cyclothiazide, and that receptors which have bound cyclothiazide are unable to desensitize (Patneau et al., 1994., Soc. Neurosci., abstract).

Our analysis shows that the first documented functional difference for the flip and flop versions of heteromeric AMPA receptors (Sommer et al., 1990)—a lower amplitude of equilibrium responses to glutamate versus kainate for A_oB_o than for A_iB_i—is not determined by the single amino acid that controls sensitivity to cyclothiazide. Rather, all of the conserved amino acids that differ between flip and flop splice variants must be exchanged to alter the relative amplitude of agonist responses to that seen for A_iB_i. Although the major part of this effect occurs upon exchange of the 5 amino acids conserved in all four AMPA receptor subunits, in GluR-B, GluR-C, and GluR-D the N-terminus of the alternatively spliced region contains an additional 2 amino acid difference conserved between flip (ThrPro) and flop (AsnAla) splice variants, while for GluR-A there is only a single amino acid difference in this region (flip, GlyPro; flop, AsnPro). Exchange of these amino acids in GluR-A and GluR-B, in conjunction with the set of 5 amino acids conserved between flip and flop splice variants of all four AMPA receptor subunits, produced an additional small increase in relative amplitude of responses to glutamate, consistent with recent experiments which indicate that multiple regions in the flip/flop module of AMPA receptors must contribute to the regulation of desensitization (Mosbacher et al., 1994; Lomeli et al., 1994).

Because cyclothiazide interferes with the process of desensitization, it is probable that high resolution analysis of responses to cyclothiazide recorded from subunit combinations additional to those examined in the present study, including those for which RNA editing is known to regulate desensitization (Lomeli et al., 1994), will reveal differences in sensitivity to allosteric regulation of desensitization beyond those described here. Also, based on differences in the extent of desensitization of responses to glutamate for AMPA receptor subunits in which the site preceding the flip/flop module is in its edited (G) or unedited (R) form, it would be expected that the relative amplitude of responses to glutamate and kainate will also be altered by RNA editing.

The experiments described here and in other studies (Partin et al., 1993, 1994) have been directed toward elucidating the structural requirements for allosteric regulation of non-NMDA receptors. Currently, no endogenous analog of cyclothiazide is known to be present in the brain, although it is known that for other receptors endogenous compounds such as neurosteroids modulate the activity of NMDA and γ-aminobutyric acid receptors (Wu et al., 1991; Paul and Purdy, 1992; Bowlby, 1993). The provocative suggestion that compounds acting as allosteric regulators of AMPA receptor activity improve performance in animal models of learning (Staubli et al., 1994; Nicoletti et al., 1992) requires further insight into the molecular identity of the binding site for cyclothiazide; such studies could in turn lead to the discovery or design of novel drugs that may improve cognitive function.

Experimental Procedures

Mutagenesis

Single and double amino acid substitutions within the flip/flop region were made by *dut- ung-* oligonucleotide-directed missense mutagenesis (Bio-Rad Muta-Gene Phagemid *In Vitro* Mutagenesis Kit [Version 2]). Mutations were made using single-stranded DNA templates generated in pBS. After mutagenesis, fragments encoding the mutated

amino acids were shuttled back into the appropriate glutamate receptor cDNAs in eukaryotic expression vectors (Keinänen et al., 1990; Sommer et al., 1990; Schall et al., 1990), using BspEI–BspEI (nucleotides 1777–2320), ApaI–MscI (nucleotides 251–2402), or BspEI–MscI (nucleotides 1777–2402) fragments as appropriate. A_i[io] was made in the eukaryotic expression vector by stepwise PCR mutagenesis, with 3′ primers encoding the point mutations and overlapping the Eco47III (nucleotide 2341) site. After subcloning into a eukaryotic expression vector (Schall et al., 1990), all mutations were confirmed by double-stranded sequencing of CsCl gradient–purified DNA. A_o[oi], B_o[ii], B_o[N-S], and B_o[oi] were made in the laboratory of P. H. Seeburg. GluR6Q755S was constructed using overlapping PCR (Ultma polymerase; Perkin-Elmer, Foster City, CA), with flanking primers covering the unique EcoRV and Eco47III sites in CMV GluR6(Q) and internal primers encoding the CAG to TCG mutation. After CsCl gradient purification of the plasmid encoding the mutation, the region that had undergone PCR amplification was sequenced.

Oocyte Electrophysiology

Oocytes were surgically obtained from anesthetized, adult Xenopus laevis (Nasco) prepared as previously described (Partin et al., 1994). Nuclear DNA injections were performed, using 1–2 μg/μl homomeric cDNA or, for hetero-oligomeric combinations, using a 1:5 ratio of GluR-A:GluR-B. Two-electrode voltage-clamp recordings were done 2–6 days after injection. In some experiments, because of the large currents expressed (1–10 μA), a virtual ground headstage was used to clamp the bath potential at ground. For these experiments, we used low-resistance agarose-cushion electrodes (Schreibmayer et al., 1994) to prevent leakage of electrolyte. The oocytes were continuously perfused with modified Barth's extracellular 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), with or without cyclothiazide (20 mM stock dissolved in dimethyl sulfoxide [DMSO]). Agonist responses were acquired by an ITC-16 interface (Instrutech Corporation) under control of the program Synapse (Synergistic Research Systems; available by anonymous FTP from Zippy.NIMH.NIH.GOV in the directory\pub\synapse) running on a MacIntosh IIfx computer. Hetero-oligomer formation was assessed using leak-subtracted ramp I–Vs from −125 mV to +50 mV, calculating the slope conductance ratio G_{+40mV}/G_{-60mV}; only oocytes with ratios > 1.0 were used for analysis.

Dose–response analysis was performed using close to maximally effective concentrations of either cyclothiazide or kainate as appropriate (see Figure 2 and Figure 3). Responses were fit to the equation:

$$I = I_{max}\{1/[1 + (EC_{50}/[ligand])^n]\},$$

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 for correction of rundown, bracketing responses to a given concentration of kainate were recorded at the beginning and end of the control dose–response run, 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 the interpolated control value. Data values are presented as mean ± SEM.

Expression of Recombinant Receptors in 293 Cells

Human embryonic kidney 293 cells (ATCC CRL 1573) were maintained in MEM with Earle's salts, 2 mM L-glutamine, and 10% fetal bovine serum (5% CO_2 at 37°C). Cultures were split upon reaching 70%–80% confluency and for transfection were plated at a density of 20,000 cells per milliter onto plastic 35 mm dishes (Nunc). After 24 hr, vectors carrying cDNAs for AMPA receptor subunits were cotransfected with a vector for the cDNA of green fluorescent protein (GFP; gift of P. H. Seeburg) using the calcium/phosphate technique described by Chen and Okayama (1987). In each case, cDNA for GFP was present at a ratio of 1:4 of the total DNA (20–40 μg/ml) added. For the expression of heteromeric receptors, cDNAs of GluR-A and GluR-B were transfected at a ratio of 1:4, respectively, to limit expression of homomeric channels from GluR-A. In some experiments, cells were replated after transfection to optimize solution exchange during concentration jump experiments.

Electrophysiological Analysis in 293 Cells

Agonist-evoked currents (holding potential = −60 mV) were recorded from isolated cells 40–72 hr after transfection using an Axopatch-200A amplifier (Axon Instruments) in whole-cell configuration, using GFP fluorescence (Chalfie et al., 1994) to identify transfected cells (450–490 nm excitation; 510 nm dichroic; 520 nm barrier). The acquisition and analysis of data were performed on a MacIntosh IIfx computer using the program Synapse (available by anonymous FTP from Zippy.NIMH.NIH.GOV in the directory\pub\synapse) to control an ITC-16 interface. The assembly of heteromeric receptors in individual cells was verified by determining the slope conductance ratio (G_{+60mV}/G_{-60mV}) for 1 mM kainate in the presence of 100 μM cyclothiazide. Only recordings from cells in which this ratio value was greater than 1 were accepted for analysis. The kinetics of onset of desensitization were fit using one exponential or the sum of two exponentials, with time constants and relative amplitudes indicated by τ_{1des}, A_1, τ_{2des}, and A_2.

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- or thin-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, 2 mM Mg–ATP, and 5 mM Cs–BAPTA (pH 7.2; osmolarity, 305 mOsm). Agonists were dissolved in normal extracellular solution and applied using a stepper motor–based fast perfusion system (Vyklicky et al., 1990). Cyclothiazide was dissolved in DMSO at 20 mM before dilution with extracellular solution (final DMSO concentration after dilution was 0.5%). Salts, biochemicals, and excitatory amino acids were purchased from Aldrich, Diagnostic Chemicals, Molecular Probes, Sigma, and Tocris Neuramin.

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