0026-895X/95/010148-07$03.00/0

MOLECULAR PHARMACOLOGY, 47:148–154, 1995.

Functional Effects of Mutations in the Putative Agonist Binding Region of Recombinant α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors

FENG LI, NEIL OWENS, and TODD A. VERDOORN

Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232

Received August 12, 1994; Accepted October 7, 1994

SUMMARY

Structural correlates of ligand binding to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors were examined by altering conserved lysine residues to either glutamate or glutamate at position 445 of the GluR-A subunit and position 449 of the GluR-B subunit. Receptors were expressed in human embryonic kidney 293 cells and in *Xenopus* oocytes as homomeric GluR-A or heteromeric combinations of GluR-A/B. The functional properties of the resulting receptors were measured with patch-clamp and voltage-clamp electrophysiology. These mutations, which reduced the total positive charge at the lysine 445 position, selectively altered the affinity of some ligands and had no obvious effect on receptor desensitization properties. The L-glutamate EC_{50} was 10.2 μM when wild-type GluR-A was coexpressed with GluR-B (GluR-A/B) and 38.9 μM when GluR-A445Q/B449Q receptors were tested. The AMPA EC_{50} was similarly reduced (wild-type, 3.71 μM; GluR-A445Q/B449Q, 21.4 μM). Receptors containing a glutamate residue at this position were even less sensitive to L-glutamate and AMPA. Whereas the apparent potency of L-glutamate varied inversely with the total positive charge regardless of the subunit, the affinity of AMPA was more sensitive to mutations in GluR-A than to those in GluR-B. Interestingly, the EC_{50} of kainate was unaffected by most of these mutations and actually increased slightly with GluR-A445E/B449E receptors. The affinity of 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) was slightly reduced with GluR-A445E/B449E receptors, whereas the apparent affinity of GYKI-52466 was unchanged. These data confirm that structural correlates of binding to AMPA receptors vary among different ligands.

Despite significant progress toward understanding the structure-function relations of recombinant glutamate receptors (1–3), the nature of the subunit domains responsible for binding ligands remains unknown. Sequence comparisons between glutamate receptor subunits and the active sites of *Escherichia coli* periplasmic amino acid-binding proteins have revealed areas of significant amino acid sequence homology in glutamate receptor subunits located in the amino-terminal portion (4–6), an area that is assumed to lie in the extracellular space. Indeed, mutations in this region have been shown either to render the resulting receptor inactive or to reduce the apparent affinity of agonists for activating the receptors (7). Such results indicate that the agonist recognition site is at least partly located in the domain that shows homology to the *E. coli* proteins. However, the relative contributions of different subunits in the heteromeric receptors were not determined in those studies, nor was the effect of the mutations on antagonist affinity. Because the measurements were made at equilibrium in *Xenopus* oocytes, it is also possible that the major effect of the mutations may be on the process of desensitization, which would remain undetected because of slow fluid exchange around the extremely large *Xenopus* oocyte.

Previously we determined the affinities of agonists and competitive antagonists for two different recombinant AMPA receptor subtypes. The most notable result of these studies was that receptors formed from a heteromeric combination of GluR-A and GluR-B subunits (GluR-A/B) showed NBQX affinities that varied markedly when different agonists were used to activate the receptors (8). One explanation for this observation was that the agonists bind to different, overlapping, substructures on the receptor and NBQX covers all of these sites. The existence of point mutations that differentially alter the affinity of agonists supports this explanation.

In the present study we have characterized the functional properties of AMPA receptors made from wild-type subunits and mutant subunits in which the conserved lysine residues

This work was supported by National Institutes of Health Grant NS30945 and The Council for Tobacco Research.

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; GluR-A, glutamate receptor type A; GluR-B, glutamate receptor type B.

normally found at position 445 of GluR-A and position 449 of GluR-B (hereafter referred to as lysine 445) were altered to either glutamine or glutamate residues. Lysine 445 was chosen based on the work of Uchino *et al.* (7), because mutations there appeared to differentially alter agonist binding. In these experiments, we wished to address the following fundamental questions. 1) Could the effects of the lysine 445 mutant be explained by alterations in desensitization behavior? 2) What is the contribution of different subunits in the binding of ligands to heteromeric receptors? 3) What is the role of the charge of this residue in establishing the affinities of ligands for the receptor? The results demonstrate that the structural correlates of binding are agonist dependent and that the GluR-A and GluR-B subunits have different roles in agonist recognition.

Experimental Procedures

Materials. AMPA was a gift from Parke Davis Research Laboratory (Ann Arbor, MI). Cyclothiazide was a gift from Eli Lilly (Indianapolis, IN). CNQX was obtained from Research Biochemicals, and all other reagents were obtained from common commercial sources.

Site-directed mutagenesis. Specific point mutations were carried out using a recombinant PCR technique (9). Two oligonucleotides, corresponding to both strands of the region in which the mutation was to be introduced, were synthesized. These oligonucleotides contained mismatched base pairs that altered the codons at the point of mutation from lysine to either glutamine or glutamate. The sequences of the forward primers were as follows: A445Q, 5′-GCGACGGCCAATATGGAGCC-3′; A445E, 5′-GCGACGGCGAATATGGAGCC-3′; B449Q, 5′-GGGGATGGCCAGTATGGGGC-3′; B449E, 5′-GGGGATGGCGAGTATGGGGC-3′. These oligonucleotides and other oligonucleotides, corresponding to flanking sequences, were used as primers to amplify by PCR the subunit DNA on both sides of the mutated region. A second PCR was performed using the two fragments generated in the first reaction and the flanking oligonucleotides. The overlap between the two original fragments primed the synthesis of the entire region, and subsequent cycles amplified this region using the flanking oligonucleotides. The mutant GluR-A PCR fragment generated was digested with *Bst*BI and *Xma*I and subcloned into the parent plasmid, which had been previously cut with the same enzymes. The GluR-B PCR products were digested with *Bst*EII and *Bsp*EI and subcloned into the parent plasmid. The entire section generated by the PCR was sequenced to confirm that the desired mutations were incorporated and that no unwanted sequence errors had been introduced.

***Xenopus* oocyte injection and electrophysiology.** Ovaries from mature *Xenopus laevis* were removed from the frogs and separated with collagenase (Sigma type II, 1.5 mg/ml). Plasmid DNAs were purified by CsCl banding, dissolved to a concentration of 100 ng/μl in water, and injected directly into the nucleus of stage VI oocytes. For heteromeric expression a ratio of 1:3 GluR-A/GluR-B cDNAs were injected, to reduce the formation of homomeric GluR-A receptors. The oocytes were incubated in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, 2.5 mM pyruvic acid, pH 7.3) for 48–72 hr before electrophysiological recording. Two-electrode voltage-clamp (Axoclamp-2A; Axon Instruments) was used to measure agonist-evoked currents in *Xenopus* oocytes. For voltage-clamp experiments, the oocytes were transferred to a Plexiglas recording chamber (volume, 200 μl) and continuously perfused with frog Ringer solution (100 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.3). Agonists and antagonists were dissolved in the Ringer solution and were applied to oocytes by switching of an eight-way Hamilton valve upstream of the recording chamber. Currents were filtered at 20 Hz (−3 db) before being digitized at 100 Hz on-line, using an Instrutech ITC-16 analog/digital converter connected to a Macintosh IIci computer.

Cell culture, transfection, and electrophysiology. HEK 293 cells were cultured, transfected, and measured essentially as described (10). After transfection by calcium phosphate precipitation, currents mediated by AMPA receptors were measured using whole-cell patch-clamp techniques. L-Glutamate was applied rapidly using a piezo-driven θ tubing pipet. Currents were filtered at 2000 Hz (−3 db) and digitized on-line at 5000 Hz with an Instrutech ITC-16 converter and a Macintosh IIfx computer.

Data analysis. Concentration-response curves were constructed by applying various concentrations of agonists and measuring the amplitude of the evoked current at equilibrium. The relationship between current and agonist concentration was fitted to the logistic equation $I = I_{max}/[1 + (EC_{50}/[agonist])^n]$. The parameters I_{max}, EC_{50}, and n were estimated for each individual cell through a computer-based iterative least-squares fitting program (IGOR; WaveMetrics). Desensitization rates were estimated by fitting current decays to single-exponential equations using IGOR. The affinity of CNQX was determined through Schild analysis (8), and the potency of GYKI-52466 was estimated by measurement of its IC_{50} for blocking currents evoked by a single concentration of L-glutamate. The means and standard errors were calculated as the logarithm of the EC_{50} or IC_{50} values. Differences between experimental groups were statistically analyzed by analysis of variance or Student's *t* test.

Results

Functional expression of both wild-type and mutant receptors was carried out in *Xenopus* oocytes and HEK 293 cells. HEK 293 cells were used to examine the desensitization characteristics of wild-type and mutant receptors. Cells expressing wild-type GluR-A/B receptors responded to 300 μM and 3 mM L-glutamate with typical peak and steady state components (Fig. 1). GluR-A445Q/B449Q receptors responded poorly to 300 μM L-glutamate (mean amplitude, 2.18 ± 0.22 pA; $n = 4$), precluding comparisons of desensitization at this agonist concentration. However, 3 mM L-glutamate evoked robust currents in these cells (amplitude, 58.5 ± 18 pA), which showed desensitization behavior indistinguishable from that of wild-type receptors. The rate of desensitization evoked by 3 mM L-glutamate (time constants of 9.80 ± 2.06 msec for mutant receptors, $n = 4$, and 10.7 ± 3.9 msec for wild-type receptors, $n = 4$) and the ratio of peak to steady state currents (mutant, 23 ± 17; wild-type, 43 ± 25) were not altered by mutations at lysine 445. Cyclothiazide (10 μM) blocked desensitization equally well in wild-type and mutant receptors, potentiating the peak current by an average of 2.8 ± 0.4-fold ($n = 4$) in cells expressing mutant receptors and 1.7 ± 0.4-fold ($n = 4$) in cells expressing wild-type receptors. Receptors containing glutamate residues at this position (GluR-A445E/B449E) were weakly sensitive to 3 mM L-glutamate but no desensitization was observed (data not shown), probably because of the reduced sensitivity to L-glutamate exhibited by these receptors.

The potency of agonists and antagonists was measured in *Xenopus* oocytes expressing homomeric and heteromeric combinations of each version of GluR-A and GluR-B. Agonist-evoked currents were readily observed in ooctyes injected with all heteromeric combinations and homomeric GluR-A and GluR-A445Q. However, homomeric GluR-A445E receptors did not appear to produce functional receptors even when tested with high concentrations (10 mM) of L-glutamate or kainate. Currents mediated by homomeric GluR-B receptors are very small in *Xenopus* oocytes (11) and were not studied here. The current densities in oocytes expressing functional subunit configurations were somewhat variable but did not differ in any systematic way between various wild-type and mutant subunit

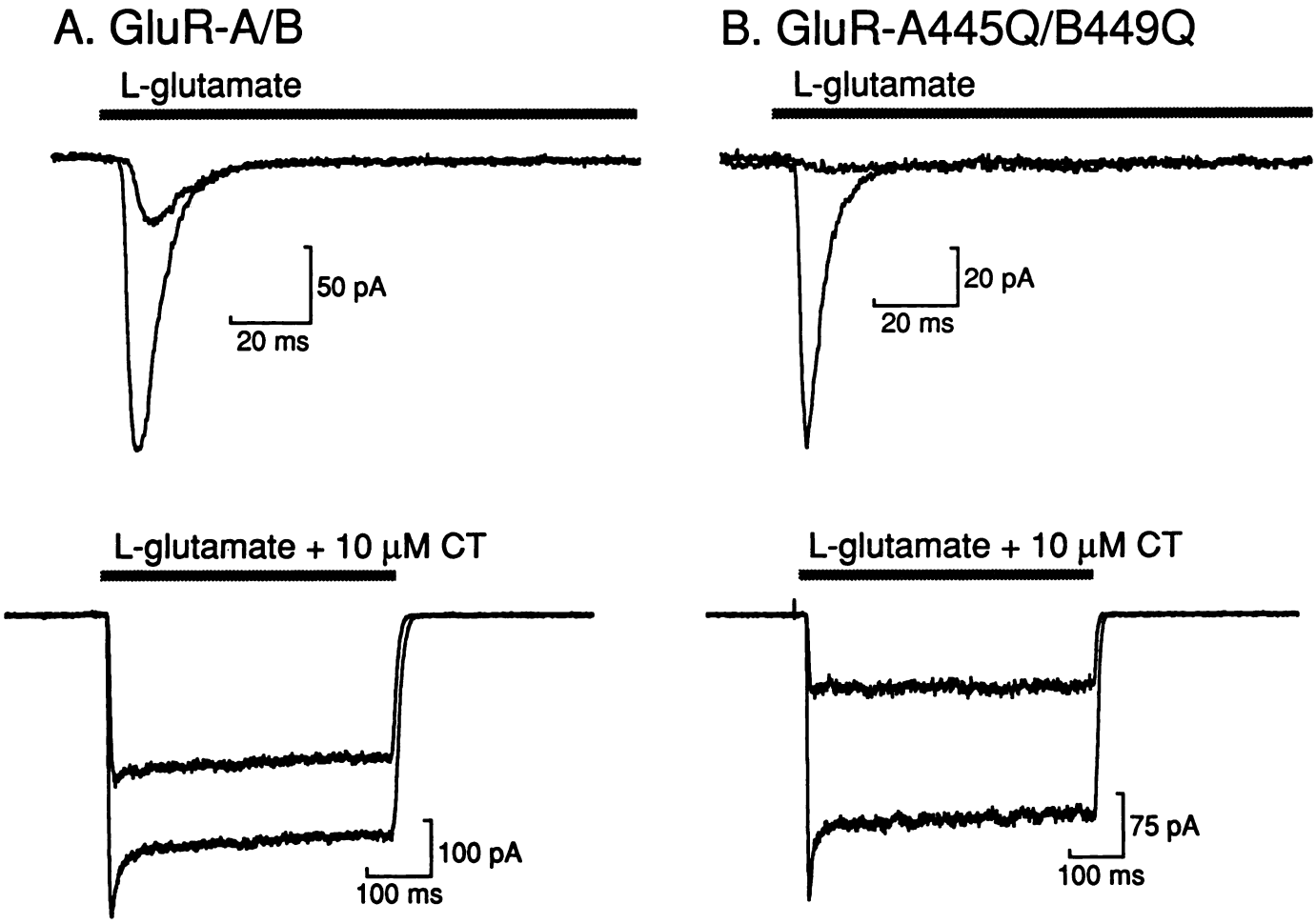

Fig. 1. Lysine 445 mutations do not alter the desensitization properties of AMPA receptors. A, *Top*, inward currents evoked by rapid application of 300 μM and 3 mM L-glutamate (during the time indicated by the *bar*) to a HEK 293 cell expressing GluR-A/B receptors (V_{hold} = −50 mV). *Bottom*, currents evoked by 300 μM and 3 mM L-glutamate in the same cell in the presence of 10 μM cyclothiazide (*CT*) (note the differences in the time scales of *top* and *bottom*). B, Results of an identical experiment conducted in a HEK 293 cell expressing GluR-A445Q/B449Q receptors. The responses to 300 μM L-glutamate were relatively smaller in this cell because of the reduced affinity of L-glutamate for these mutant receptors.

combinations. Thus, the mutations did not significantly reduce the ability of the subunits to form functional receptors.

Concentration-response relations were constructed to estimate the affinity of agonists at wild-type and mutant receptors. The results of this analysis are summarized in Table 1. Heteromeric receptors consisting of two wild-type (GluR-A/B) or mutant (GluR-A445Q/B449Q or GluR-A445E/B449E) subunits showed typical behavior in these experiments (Fig. 2). The potencies of L-glutamate and AMPA were reduced by these amino acid substitutions. Importantly, the concentration-response curves for kainate were identical for wild-type and GluR-A445Q/B449Q receptors and the kainate potency was increased in cells expressing GluR-A445E/B449E receptors. The agonist-specific effects of the mutations indicate that changes in lysine 445 do not cause generalized loss of receptor function. These results also support a role for lysine 445 in agonist recognition.

To examine the relative roles of GluR-A and GluR-B in forming the binding site for AMPA and L-glutamate, we characterized heteromeric receptors in which the subunits carried different residues at position 445. The potency of L-glutamate systematically decreased as the number of residues carrying a negative charge at lysine 445 increased (Fig. 3A), assuming that GluR-A and GluR-B assemble with approximately 1:1 stoichiometry. The particular subunit that carried the mutation made no difference for the apparent affinity of L-glutamate. However, the affinity of AMPA was much more sensitive to alterations of the GluR-A subunit than to mutations of GluR-B (Fig. 3B). This was clearly observed when glutamate-containing mutants were used. The AMPA affinity was slightly lower than control for GluR-A/B449E receptors but was markedly reduced for GluR-A445E/B receptors. Although the disruption of AMPA affinity was less marked in receptors containing one glutamine mutant, the trend remained similar, with mutations in GluR-A showing a larger effect than those in GluR-B.

The affinity of mutant receptors for antagonists was also examined. Schild analysis was used to estimate the affinity of the competitive AMPA receptor antagonist CNQX for mutant and wild-type receptors. In each case the slope of the regression was 1, indicating that CNQX blocked each receptor type competitively. Using kainate as the agonist, the mean CNQX pA_2 of wild-type GluR-A/B receptors was 6.45 ± 0.06 (K_b = 355 nM, n = 9), compared with 6.35 ± 0.04 (K_b = 446 nM, n = 9) for GluR-A445Q/B449Q receptors and 6.25 ± 0.06 (K_b = 562

TABLE 1

Effects of mutations on agonist potencies

Values were derived from concentration-response curves constructed in individual *Xenopus* oocytes expressing each of the wild-type and mutant receptors. Data from each experiment were fitted to the logistic equation (see *Experimental Procedures*), and the means and confidence intervals were calculated from the logarithm of the EC_{50} estimates.

Receptor type	EC_{50}		
	L-Glutamate	AMPA	Kainate
		μM	
A	8.51 (4.47–16.5) ($n = 4$)	6.76 (1.38–33.1) ($n = 3$)	16.2 (10.5–25.1) ($n = 5$)
A445Q	24.0 (15.4–37.1)[a] ($n = 4$)	347 (18.6–63,000)[a] ($n = 3$)	15.1 (10–22.9) ($n = 7$)
A/B	9.12 (4.17–19.5) ($n = 6$)	3.71 (2.88–4.68) ($n = 5$)	46.8 (38.0–58.9) ($n = 5$)
A445Q/B	11.5 (7.59–17.4) ($n = 7$)	5.37 (1.82–15.5) ($n = 7$)	32.4 (24.5–42.7)[b] ($n = 7$)
A445E/B	30.2 (10.0–91.2)[c] ($n = 7$)	51.3 (8.91–295)[b] ($n = 7$)	40.7 (24.0–100) ($n = 7$
A/B449Q	10.0 (4.37–23.4) ($n = 9$)	2.14 (0.955–4.79) ($n = 8$)	31.6 (19.5–50.1) ($n = 5$)
A/B449E	30.1 (6.46–141) ($n = 7$)	6.17 (1.51–25.1) ($n = 6$)	55.0 (37.2–81.3) ($n = 8$)
A445Q/B449Q	38.9 (25.7–57.5)[b] ($n = 8$)	21.4 (17.4–25.7)[b] ($n = 6$)	38.9 (25.7–57.5) ($n = 8$)
A445E/B449E	562 (447–692)[b] ($n = 9$)	6,460 (603–67,600)[b] ($n = 4$)[d]	17.8 (12.3–25.1)[b] ($n = 4$)

[a] Significantly different from wild-type GluR-A ($p < 0.05$).
[b] Significantly different from wild-type GluR-A/B ($p < 0.05$).
[c] Marginally different from wild-type GluR-A/B ($p = 0.05$).
[d] Data estimated from incomplete concentration-response curves.

nM, $n = 7$) for GluR-A445E/B449E receptors. The pA_2 for the glutamate-containing mutant receptors was significantly less than that for controls ($p < 0.05$), indicating that this amino acid residue also plays some role in CNQX binding. The difference between wild-type and GluR-A445Q/B449Q receptors was not significant. Qualitatively similar results were also seen when L-glutamate was used as an agonist. GluR-A445Q/B449Q gave a pA_2 of 6.37 ± 0.08 ($n = 3$), and with GluR-A445E/B449E receptors the pA_2 dropped to 6.03 ± 0.03 ($n = 3$). This compares with a pA_2 of 6.23 found previously for wild-type GluR-A/B receptors (8).

In contrast, the potency of the noncompetitive antagonist GYKI-52466 appeared to be unaffected by changes in lysine 445. Increasing concentrations of GYKI-52466 were used to block the current evoked by 300 μM kainate in oocytes expressing GluR-A/B and GluR-A445E/B449E receptors. The IC_{50} of GYKI-52466 was 43.6 μM (95% confidence interval, 22.4–83.2 μM; $n = 4$) with wild-type receptors and 64.6 μM (95% confidence interval, 38.9–107 μM, $n = 4$) with GluR-A445E/B449E receptors. These values were not significantly different from each other ($p > 0.1$).

Discussion

We have examined the functional consequences of mutating a conserved lysine residue (position 445 in GluR-A and position 449 in GluR-B) in two AMPA receptor subunits using different expression systems. The desensitization behavior of wild-type and mutant receptors was compared using rapid application of agonists to transfected HEK 293 cells, and equilibrium pharmacological studies were carried out in *Xenopus* oocytes. The results reveal the important role of lysine 445 in ligand recognition of and binding to AMPA receptors. The present work confirms and extends previous studies in which the GluR-A445E mutant was tested for agonist potency in *Xenopus* oocytes (7).

The rapid desensitization typically evoked by L-glutamate and AMPA cannot be observed in *Xenopus* oocytes because of slow solution changes around such large cells. The currents observed in oocytes arise from a desensitized state of AMPA receptors, and the EC_{50} values reported here represent the apparent affinities of the desensitized receptor. Changes in the desensitization behavior induced by lysine 445 mutations could cause shifts in the concentration-response curves that bear little relationship to agonist binding. Using rapid agonist application in HEK cells, we observed no obvious differences in time course or extent of desensitization between wild-type and GluR-A445Q/B449Q receptors. In contrast, changes in the concentration dependence of desensitization due to reduced agonist affinity were seen. This suggests that alterations in function produced by the mutations of lysine 445 involve the affinity of both resting and desensitized receptors.

There is also the possibility that these mutations modify the reaction leading to channel opening from an agonist-bound closed state. Changes in the equilibrium of this step may shift the concentration-response relationship even though no alterations of agonist affinity occurred. Although we cannot completely rule out this possibility, some of the results argue against this alternative explanation. Changes in the channel opening step would likely change the kinetics of channel opening, closing, and desensitization observed in the fast application experiments. No such changes were seen, but single-channel kinetic measurements would be necessary to completely examine this issue. More importantly, the mutation also affected the affinity of a competitive antagonist, CNQX. Because antagonists do not open the channel, the open/closed equilibrium argument cannot account for changes in CNQX affinity caused by lysine 445 mutations. Therefore, the simplest explanation for the

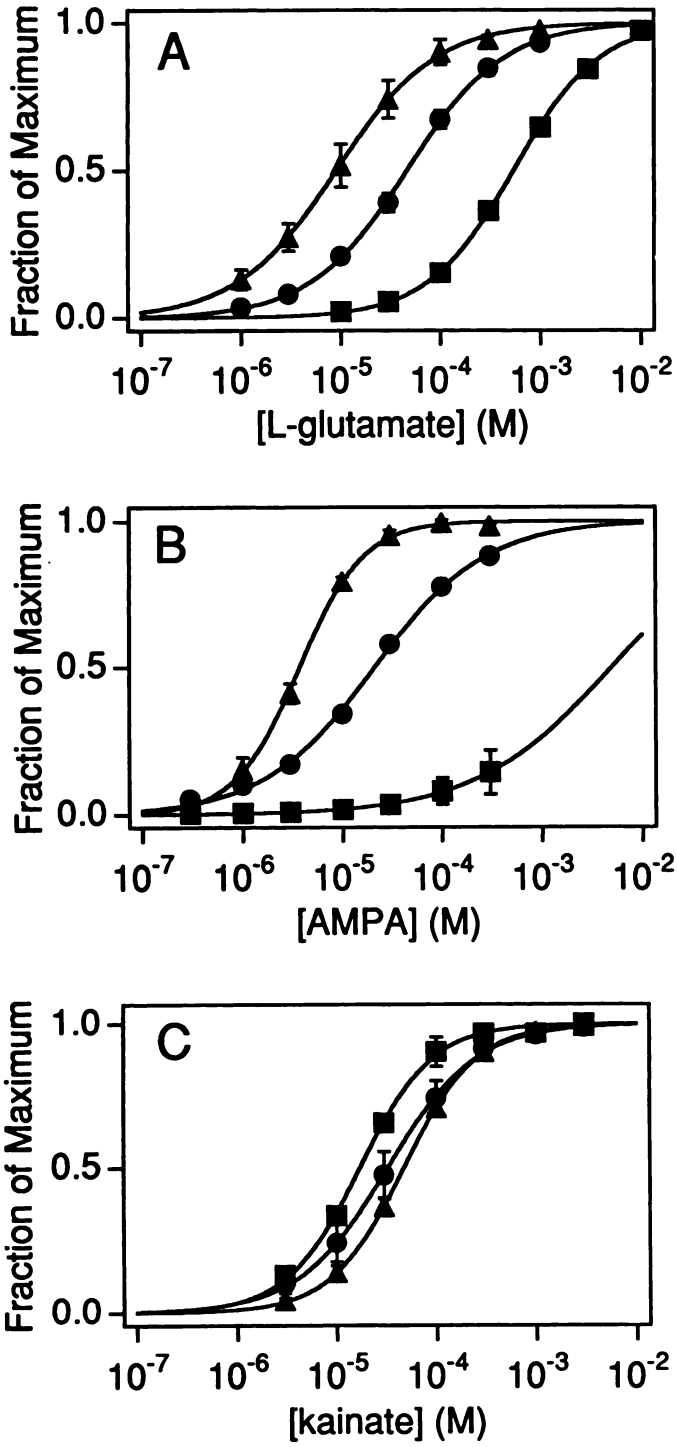

Fig. 2. Mutation of lysine 445 systematically reduces the apparent affinity of L-glutamate and AMPA but not kainate. Concentration-response curves were generated in *Xenopus* oocytes expressing wild-type (▲), GluR-A445Q/B449Q (●), and GluR-A445E/B449E (■) receptors. The *points* represent the mean ± standard error of the fraction of maximum measured in four to nine cells, using L-glutamate (A), AMPA (B), and kainate (C) as agonists.

present results is that lysine 445 forms part of the agonist and antagonist binding pocket on AMPA receptors.

The mutations studied here apparently altered the binding of AMPA receptor ligands without markedly changing other receptor properties, impeding subunit assembly, or causing generalized disruption of functionality. The ion flow properties were likewise unaffected by the mutations. In HEK 293 cells and *Xenopus* oocytes, the current-voltage curves of wild-type and mutant receptors containing GluR-A and GluR-B were linear or outwardly rectifying (data not shown) and did not differ with subunit combination. Thus, the mutations apparently do not alter the assembly of subunits into heteromeric complexes, because current-voltage curve rectification depends on assembly of GluR-A and GluR-B subunits (12, 13). Homomeric GluR-B receptors mediate very little current when expressed in *Xenopus* oocytes (11) and would not be expected to contribute to the currents observed here. Some oocytes injected with cDNAs encoding GluR-A and GluR-B subunits (or mutants) could have expressed a small proportion of homomeric GluR-A receptors because of incomplete assembly of the two subunits. Differences in the EC_{50} values for L-glutamate and AMPA between homomeric GluR-A445Q receptors and heteromeric assemblies containing GluR-A445Q indicate that the relative amounts of homomeric GluR-A receptors in these oocytes must have been quite small. The current density varied with transfection (HEK 293 cells) or injection (oocytes) but did not systematically vary with subunit combination. Moreover, the affinity of kainate was not reduced and in some cases was increased when mutant receptors were expressed. These last two observations preclude the possibility that the mutations caused nonspecific generalized loss of functionality.

Earlier pharmacological characterization of different recombinant AMPA receptors revealed that GluR-A/B receptors showed affinities for the competitive antagonist NBQX that differed according to which agonist was used to activate the receptors (8). One interpretation of the agonist-dependent NBQX affinity is that the agonists bind to different substructures within the subunits. The current observations provide support for this interpretation. Clearly, the structure of the binding pocket for L-glutamate and AMPA is altered by replacement of the lysine with glutamine or glutamate. In contrast, the structures that bind kainate are markedly less affected by the mutations. This indicates that different substructures of AMPA receptors are responsible for binding these ligands.

The differential effects of mutations in GluR-A and GluR-B further support this conclusion and demonstrate that the AMPA and L-glutamate binding pockets must also be structurally distinct, at least in heteromeric GluR-A/B receptors. Mutations in GluR-A were markedly more effective at reducing the AMPA affinity than were mutations in GluR-B. The potency of L-glutamate did not depend on the subunit that carried mutation. It appears that the L-glutamate binding pocket may be formed by both GluR-A and GluR-B in heteromeric GluR-A/B receptors, whereas the AMPA binding pocket is dominated by the GluR-A subunit.

The L-glutamate potency seemed to correlate inversely with the number of residues carrying a positive charge at lysine 445. If one assumes that heteromeric receptors contain the same number of GluR-A and GluR-B subunits, then the total charge at this position can be estimated from the charges of the amino acids contributed by each subunit. According to this assumption, EC_{50} values derived from heteromeric GluR-A/B receptors carrying the same total charge were pooled and subjected to statistical analysis. With the exception of comparisons at the far ends of the continuum (−2 versus −1 and 2 versus 1), all pairwise comparisons showed significant differences ($p < 0.01$),

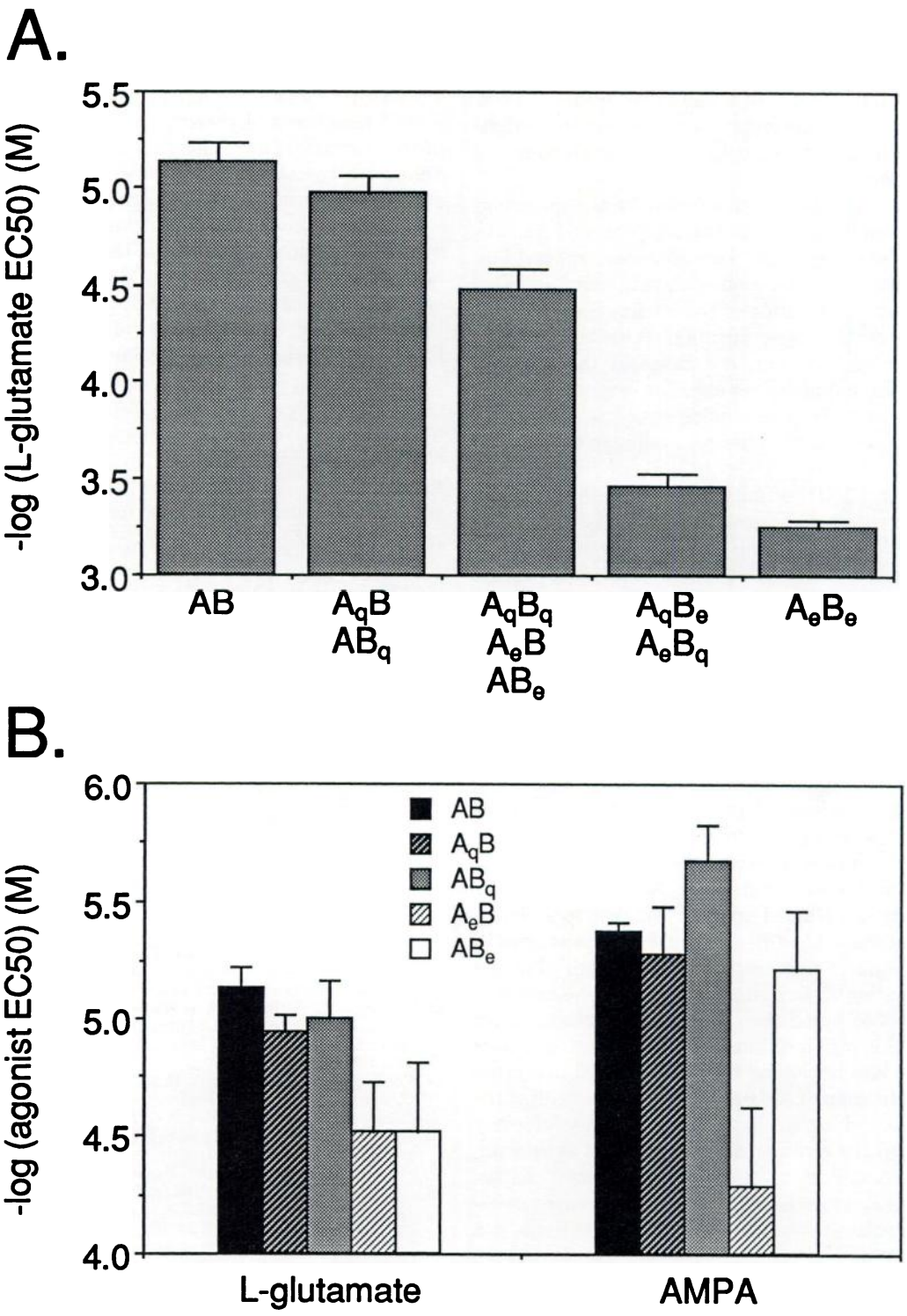

Fig. 3. Lysine 445 mutants differentially affect AMPA and L-glutamate potencies. A, This bar graph summarizes the effects of total charge at this amino acid position on the EC_{50} of L-glutamate in heteromeric GluR-A/B receptors. Data from different mutants carrying the same total charge (assuming a 1:1 ratio of GluR-A and GluR-B in the receptor) at lysine 445 were pooled to derive the values (mean ± standard error) presented here. Each *bar* is labeled according to which heteromeric complexes contributed the data. B, This bar graph illustrates the different roles of GluR-A and GluR-B mutants in altering the AMPA EC_{50} in heteromeric GluR-A/B receptors. The values are mean ± standard error of log (EC_{50}) for the indicated receptor types. Note the differences between GluR-A mutants and GluR-B mutants with respect to AMPA and the lack of differences when L-glutamate was examined.

suggesting that the total charge at lysine 445 is an important determinant of L-glutamate affinity at recombinant AMPA receptors. Thus, L-glutamate binding likely involves a direct electrostatic interaction with this particular lysine residue. However, the possibility that mutation of this residue disrupts some local secondary structure cannot be completely ruled out by the present observations.

Interestingly, the agonists most affected by the mutations are those that strongly desensitize the receptor, and kainate, which does not apparently cause desensitization, showed limited changes in potency associated with this mutation. One might speculate that the location of the binding pocket on the receptor determines the desensitization behavior produced upon agonist binding. However, the fact that the lysine to glutamine switch did not appear to alter the desensitization of the receptors indicates that this binding region is not directly involved in the conformational changes leading to desensitization.

The reduction in CNQX affinity observed with the GluR-A445E/B449E receptors indicates that lysine 445 (GluR-A) also plays some role in recognition of this competitive antagonist. It will be of interest to examine the kinetics of CNQX binding and unbinding, to determine which of these properties is altered in the mutant receptors. The potency of the noncompetitive antagonist GYKI-52466 was similar with wild-type and GluR-A445E/B449E receptors, suggesting that lysine 445 plays little role in forming the GYKI-52466 binding site.

A great deal of speculation has focused on the amino acid sequence homology between bacterial periplasmic amino acid-binding proteins and regions of glutamate receptor subunits (4–6). It has been postulated that these homologous regions may form structures responsible for agonist binding in glutamate receptors. This postulate has particular significance because the three-dimensional structures of the bacterial proteins have been elucidated. Guided in part by this hypothesis, Kuryatov *et al.* (14) have identified a number of amino acids in the *N*-methyl-D-aspartate receptor type 1 subunit that are involved in the binding of the coagonist glycine to this receptor. Uchino *et al.* (7) also based their original mutagenesis work with GluR-A on this region of homology and found a number of amino acid residues (including the one examined here) that disrupt agonist activation of AMPA receptors. Nearly all of the mutations they tested either reduced the affinity of every agonist or rendered the receptor nonfunctional. It is thus unclear whether such mutations alter specific interactions between amino acids and agonists or whether they cause general disruption of the subunit secondary structure. The lysine 445 to glutamate mutation was the only one examined by Uchino and co-workers that altered agonist affinity selectively. Notably, lysine 445 falls outside of the regions postulated by O'Hara *et al.* (5) and Cockcroft *et al.* (6) to have the greatest structural similarity to the bacterial binding proteins.

The present results suggest that lysine 445 is critically involved in agonist binding and recognition in recombinant AMPA receptors and, therefore, more detailed characterization of the functional role of this amino acid residue is justified. We are currently examining the effects of the mutations on the affinities of other ligands, including quisqualate and NBQX. It will also be of interest to characterize receptors carrying other amino acids, such as arginine, at this position. Possible alteration of agonist cooperativity by these mutations is also being investigated. The present results, along with these future studies, will provide a clearer picture of the structural correlates of ligand binding to AMPA receptors.

Acknowledgments

The authors thank Drs. Tina Johansen, C. David Weaver, and David Lovinger for helpful discussions. The gifts of AMPA from Parke Davis and cyclothiazide from Eli Lilly are gratefully acknowledged.

References

1. Nakanishi, S. Molecular diversity of glutamate receptors and implications for brain function. *Science (Washington D. C.)* **258**:597–603 (1992).
2. Hollmann, M., and S. Heinemann. Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17**:31–108 (1994).
3. Seeburg, P. H. The *TiPS/TINS* Lecture: the molecular biology of mammalian glutamate receptor channels. *Trends Pharmacol. Sci.* **14**:297–303 (1993).
4. Nakanishi, N., N. A. Shneider, and R. Axel. A family of glutamate receptor genes: evidence for the formation of heteromultimeric receptors with distinct channel properties. *Neuron* **5**:569–581 (1990).
5. O'Hara, P. J., P. O. Sheppard, H. Thogersen, D. Venezia, B. A. Haldeman, V. McGrane, K. M. Houamed, C. Thomsen, T. L. Gilbert, and E. R. Mulvihill. The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11**:41–52 (1993).
6. Cockcroft, V. B., M. O. Ortells, P. Thomas, and G. G. Lunt. Homologies and disparities of glutamate receptors: a critical analysis. *Neurochem. Int.* **23**:583–594 (1993).
7. Uchino, S., K. Sakimura, K. Nagahari, and M. Mishina. Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor channel. *FEBS Lett.* **308**:253–257 (1992).
8. Stein, E., J. A. Cox, P. H. Seeburg, and T. A. Verdoorn. Complex pharmacological properties of recombinant α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subtypes. *Mol. Pharmacol.* **42**:864–871 (1992).
9. Higuchi, R. Recombinant PCR, in *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.). Academic Press, San Diego, CA, 177–183 (1992).
10. Verdoorn, T. A. Formation of heteromeric γ-aminobutyric acid type A receptors containing two different α subunits. *Mol. Pharmacol.* **45**:475–480 (1994).
11. Boulter, J., M. Hollmann, A. O'Shea-Greenfield, M. Hartley, E. Deneris, C. Maron, and S. Heinemann. Molecular cloning and functional expression of glutamate receptor subunit genes. *Science (Washington D. C.)* **249**:1033–1037 (1990).
12. Verdoorn, T. A., N. Burnashev, H. Monyer, P. H. Seeburg, and B. Sakmann. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science (Washington D. C.)* **252**:1715–1718 (1991).
13. Hume, R. I., R. Dingledine, and S. F. Heinemann. Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science (Washington D. C.)* **253**:1028–1031 (1991).
14. Kuryatov, A., B. Laube, H. Betz, and J. Kuhse. Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. *Neuron* **12**:1291–1300 (1994).

Send reprint requests to: Todd A. Verdoorn, Department of Pharmacology, Vanderbilt University, Nashville, TN 37232-6600.