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A Venus Flytrap Mechanism for Activation and Desensitization of α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid Receptors*

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Desensitization of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype of glutamate receptor channels is an important process shaping the time course of synaptic excitation. Upon desensitization, the receptor channel closes and the agonist affinity increases. So far, the nature of the structural rearrangements leading to these events was unknown. On the basis of the structural homology of the ligand binding domains of AMPA receptors and of the bilobated bacterial periplasmic proteins, we now show that agonist interaction with one lobe of the GluR1 subunit of homomeric AMPA receptors controls channel activation while additional interactions with the other lobe cause channel desensitization. Accordingly, we suggest that the transition of the AMPA receptor channel to the desensitized state involves the agonist-mediated stabilization of the closed lobe conformation of its binding domain and is a process akin to that used by the venus flytrap.

The AMPA[1] subtype of glutamate receptors plays a central role in brain excitatory synaptic transmission. Activated by agonists, the receptor channels first open and then desensitize (1–4). The process of desensitization is of physiological importance since it may determine both the duration of excitatory postsynaptic currents as well as the ability to respond to high frequency stimulation (3, 5-10). Desensitization results from a transition, still unexplained in structural terms, of the receptor channel complex from an open state, which binds the agonist with low affinity, to a closed state, which binds with high affinity (11–15). Recently, it was suggested (16–19) that the structure of the agonist binding domain of glutamate receptor channels is homologous to that of bacterial periplasmic binding proteins and in particular to the lysine-arginine-ornithine binding protein (LAOBP) (20). For this bi-lobated amino acid binding protein, a substrate binding mechanism akin to that of a venus flytrap was suggested. A single substrate molecule binds to lobe I with low affinity and, by further binding to lobe II, stabilizes a high affinity closed lobe conformation (21–23). Assuming that the structural homology between AMPA receptors and bacterial periplasmic binding proteins extends to a similarity in function, we have investigated here whether the elevation of agonist affinity upon AMPA receptor desensitization might also involve a venus flytrap mechanism. The data presented here strongly suggest that this might indeed be the case.

EXPERIMENTAL PROCEDURES

Mutagenesis and Oocyte Expression—GluR1 amino acid residues possibly involved in agonist binding were identified on the basis of their homology with the residues lining the ligand binding pocket of LAOBP (23), as deduced from amino acid sequence alignments of glutamate receptors (from rat), kainate binding proteins, and bacterial periplasmic proteins (LAOBP, QBP, and HisJ) using the UW-GCG sequence analysis software package (16–18). Minor shifts were introduced to optimize the homology. The selected residues were mutated in order to induce only moderate changes of the chemical nature and bulkiness of the amino acid side chains. Multiple mutagenesis was performed using degenerate primers and pSK-GluR1-derived (24) uridine-containing single-stranded DNA (25). A small fragment containing the mutation was sequenced, excised with restriction enzymes, and used to replace the corresponding fragment of a plasmid carrying wild-type (WT) GluR1 cDNA flanked by the 5′- and 3′-untranslated regions of globin cDNA (pBTG-WT GluR1). Capped cRNA was transcribed using an Ambion MEGAscript kit. *Xenopus* oocytes were injected with 10 ng of cRNA and analyzed 3–14 days later by standard two-electrode voltage clamp methods. Agonists were applied by perfusion. Oocytes clamped at −80 mV were screened for kainate (KA)-induced currents (up to 10 mM), and then increasing concentrations of quisqualate (QA) and glutamate (Glu) were used to desensitize and thereby inhibit the responses induced by co-applied 100 μM KA. When QA was used to induce peak and steady-state currents (Fig. 2), 100 μM CYZ alone was applied ~30 s prior to the co-application of QA + CYZ.

Data Analysis—Dose-response curves were usually analyzed on 5–10 oocytes, usually from more than one batch. Normalized and average data were fitted by the Sigmaplot software to the logistic equation (11): fractional response = $(A - D)/(1 + (C/\text{agonist concentration})^B) + D$, where A and D are, respectively, asymptotic maximum and minimum (approaching 1 and 0, respectively, for KA dose-response curves), B is the slope at the inflection point (representing the Hill coefficient, negative slopes used for inhibition curves), and C is the agonist concentration at the inflection point (EC_{50} for KA dose-response curves or IC_{50} for inhibition curves). IC_{50} values for QA and Glu were corrected according to the relevant KA EC_{50} to derive IC_{50}' values as described (12): $IC_{50}' = IC_{50}/(1 + (A/EC_{50}))$, where IC_{50} is the concentration of desensitizing agonist needed to inhibit 50% of the response of the non-desensitizing agonist, A is the concentration of the non-desensitizing agonist used in the experiment (in our case, 100 μM KA), and EC_{50} is the concentration of the non-desensitizing agonist needed to give half-maximal response. For curves describing apparent activation and desensitization of QA responses in the presence of CYZ (Fig. 2), curve fitting was performed as above. The data describing the steady-state phase of these currents could be fitted by an equation that is the product of the equation fitted to peak currents data and the equation fitted to steady-state/peak currents data.

RESULTS

To identify the ligand binding residues and confirm that the AMPA receptor agonist binding domain has a bi-lobated structure similar to that of LAOBP, we substituted by site-directed mutagenesis in the GluR1 (flop) subunit of the AMPA receptor

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[1] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; LAOBP, lysine-arginine-ornithine binding protein; WT, wild-type; KA, kainate; QA, quisqualate; Glu, glutamate; CYZ, cyclothiazide.

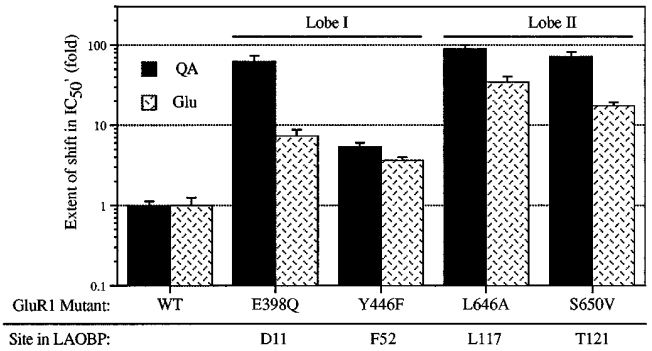

FIG. 1. **Effect of mutagenesis of GluR1 residues aligning with the ligand binding residues of LAOBP on the ability of QA and Glu to inhibit KA-evoked currents.** Amino acid substitutions in GluR1 are indicated in one-letter code, and their positions are numbered according Hollmann *et al.* (24). *Bars* represent the magnitude of the shift (in folds) of the QA and Glu IC_{50}' values, measured for the mutants and compared with those of WT GluR1 (taken as 1.0). For WT GluR1, the actual IC_{50}' values are 0.31 μM for QA and 22 μM for Glu. KA EC_{50} values displayed by the mutants were similar to that of WT GluR1. Alignment to LAOBP residues participating in ligand binding is presented below the GluR1 positions, and assignment to lobes (17) is shown above the *bars*. *Error bars* represent S.E.

(24, 26), the amino acids at residues that align with the ligand binding residues in lobes I and II of LAOBP. Expressing the GluR1 homomeric mutants in *Xenopus* oocytes, we observed (Fig. 1) that mutations E398Q and Y446F in lobe I (amino acids 387–491 and 727–770 as defined by Stern-Bach *et al.* (17)) and L646A and S650V in lobe II (amino acids 499–504 and 629–724 as defined by Stern-Bach *et al.* (17)) very significantly affect the ability of QA and Glu to desensitize the GluR1 channel and to inhibit thereby the responses induced by the weakly desensitizing agonist KA (12, 27). The KA EC_{50} values were not significantly changed by these mutations. Several other mutations did not result in detectable KA-induced currents (see below). We thus interpret these results as suggesting that residues Glu-398, Tyr-446, Leu-646, and Ser-650, which reside in lobes I and II, form part of a binding domain that accommodates QA and Glu and allows these ligands to exert their action as desensitizing agonists. These results provide further support to the extracellular location of the loop bordered by the putative transmembrane segments III and IV (for reviews describing the debate concerning glutamate receptor topology see Refs. 28–30) and confirm the suggested similarity of architecture of the ligand binding domain of LAOBP and GluR1 (16–18).

To determine whether this structural homology extends to a similarity in function, we investigated the role of the individual lobes of the agonist binding domain of GluR1. By analogy to LAOBP, we examined whether the low affinity open channel state of GluR1 involves agonist interactions with amino acid residues in lobe I, whereas the high affinity desensitized state involves additional interactions with lobe II. We therefore studied the effects of the desensitizing agonist QA on WT GluR1, E398Q (lobe I), L646A, and S650V (lobe II) GluR1 mutants. In the presence of CYZ, a drug used to reduce the rate of desensitization (31) and whose apparent affinity was unaffected in all GluR1 mutants tested here (data not shown), QA produced a transient peak current soon followed by a decay to a steady-state current (see current traces in Fig. 2). Although the full amplitude of the transient peak current is not recorded, one can nevertheless relate this transient current to channel activation, while the extent of decay to the steady-state current is evidently the result of a desensitization process. We used these parameters to distinguish between the activation and desensitization phases and detect qualitative differences between lobe I and lobe II mutants.

In Fig. 2, we monitored the amplitudes of the transient peak and steady-state currents, as well as their ratios (reflecting the apparent extent of desensitization) at increasing QA concentrations. Comparing the normalized dose-response curves and the current traces of the GluR1 E398Q lobe I mutant to those of WT GluR1, one notices a systematic loss of sensitivity to QA and a rightward shift of the dose-response curves. The simplest explanation for this behavior is that the E398Q lobe I mutation has weakened the agonist binding interaction and thus caused a reduced receptor occupancy for any given QA concentration. In contrast, the dose dependence of the steady-state/peak current ratios as well as the shapes of the QA-evoked current traces of the S650V lobe II mutant clearly differ from those of WT GluR1 and the E398Q lobe I mutant and express a marked decrease in the apparent extent of desensitization. The L646A lobe II mutant displays a behavior similar to that of the S650V lobe II mutant (data not shown), as in both mutants an apparent desensitization to only ~60% of the peak current is produced at high QA concentration (compared to ~23 and ~27% for the WT GluR1 and the E398Q mutant, respectively).

In view of the mechanism proposed above for activation and desensitization of AMPA receptors, is was also of interest to locate the binding site for the weakly desensitizing agonist KA. The mutations E398Q, Y446F, L646A, and S650V caused no elevation in the KA EC_{50} values, while several other mutations at residues that align with LAOBP ligand binding residues (GluR1 Y446V, T476A, E701A, E701T tested here and R481K tested by Uchino *et al.* (32)) resulted in a lack of oocyte-mediated expression of KA-induced responses. However, the mutations E398L and Y401A (the expression of the latter was detectable only as a heteromeric complex of GluR1 with the otherwise practically silent wild type GluR2) reduced the agonist potency of KA and caused a shift in the KA EC_{50} by a factor of 3–4 compared to homomeric WT GluR1 or heteromeric WT GluR1 + WT GluR2 (data not shown). Since a mutation equivalent to GluR1 Y446V, performed in the chick kainate binding protein, a member of the glutamate receptor family, has established the crucial role played by this tyrosine residue in KA binding (33), we infer that KA is likely to interact with a domain in lobe I including residues Glu-398, Tyr-401, and Tyr-446 to produce channel activation.

Interestingly, studies of Leu-646 GluR1 mutants (corresponding to Leu-117 in lobe II of LAOBP) showed that strengthening the interaction of KA with this residue in lobe II was paralleled by an increased KA-induced desensitization. The GluR1 mutants L646V and L646T (but not L646A) were found to exhibit KA EC_{50} values, which were 6-fold (data not shown) and 20-fold (Fig. 3*A*), respectively, lower than that of WT GluR1 (in contrast, QA IC_{50}' values in these mutants were 3- and 34-fold higher, respectively, than that of WT GluR1). These results suggest that the KA binding to the GluR1 L646V and L646T mutants involves an interaction with a site in lobe II that does not participate normally in KA binding to WT GluR1. To ascertain that this interaction with lobe II affects the desensitization process as expected, we investigated whether the GluR1 L646T mutant displays an increased KA-induced desensitization. Since in the oocyte system no KA-induced transient peak currents were observed even in the presence of CYZ, we monitored the CYZ-elicited potentiation of the steady-state KA currents, as this potentiation was suggested to be due to a CYZ-mediated relief of a previously undetected KA-induced desensitization (34). As seen in Fig. 3*B*, CYZ was found to enhance the KA responses of the L646T mutant to a much larger extent than that observed for the WT GluR1 or the L646A GluR1 mutant, suggesting that prior to

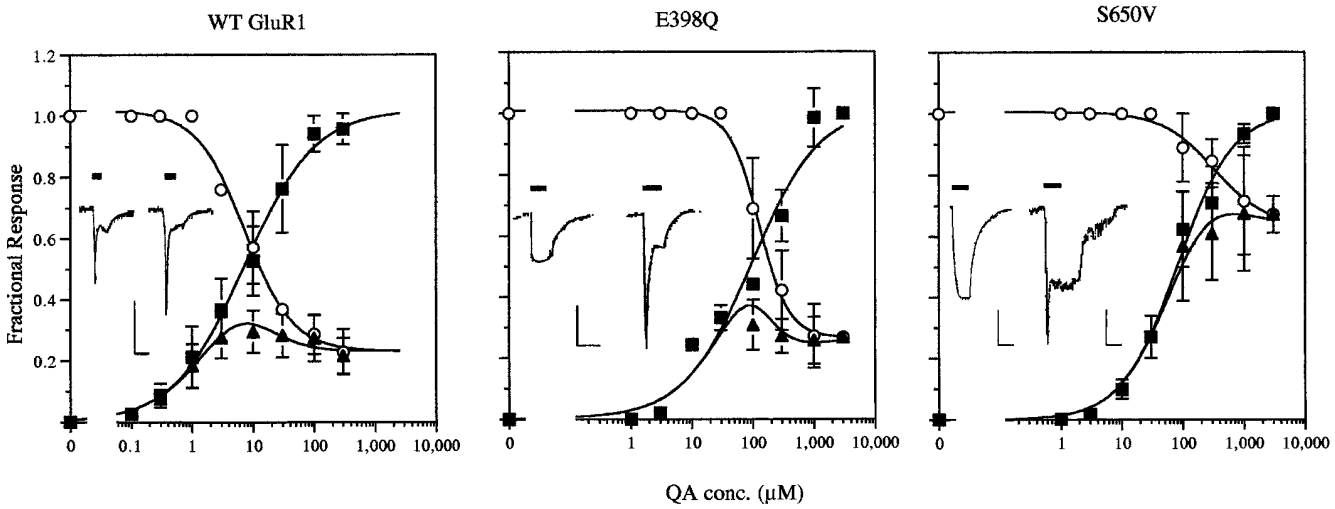

FIG. 2. **QA dose-response curves of WT, lobe I, and lobe II GluR1 mutants.** Normalized peak currents (*rectangles*, apparent activation), steady-state currents (*triangles*), and steady-state/peak currents (*circles*, apparent desensitization) measured from oocytes expressing WT GluR1, the lobe I mutant E398Q, and the lobe II mutant S650V are plotted as a function of QA concentrations at a constant CYZ concentration of 100 μM. *Error bars* represent S.D. for groups of 3 or 4 oocytes. In all current trace inserts, *solid horizontal bars* above traces represent QA application, and *horizontal bars* below traces represent a time scale of 1 min. For WT GluR1, current trace inserts describe responses of an oocyte to application of 30 μM (*left trace*) and 300 μM (*right trace*) QA, and the *vertical scale bar* corresponds to a current amplitude of 500 nA. Current traces for the E398Q mutant describe responses of an oocyte to application of 30 μM (*left trace*) and 300 μM (*right trace*) QA, and the *vertical scale bar* corresponds to a current amplitude of 50 nA. In S650V, the *left trace* describes a response of an oocyte to 300 μM QA (current amplitude scale bar is 125 nA), and the *right trace* describes a response of another oocyte to 3,000 μM QA (current amplitude scale bar is 250 nA). QA EC_{50} and IC_{50} for WT GluR1 are 7.1 μM and 13 μM, respectively, and the asymptotic minimum calculated from curve fitting for the apparent desensitization curve is 0.232. In E398Q, the QA EC_{50} and IC_{50} values are elevated 13.6- and 15-fold, respectively, compared to WT GluR1, while the asymptotic minimum is similar to that of WT GluR1. In S650V, the QA EC_{50} value is 11.5-fold that of WT GluR1 and the asymptotic minimum is 0.634.

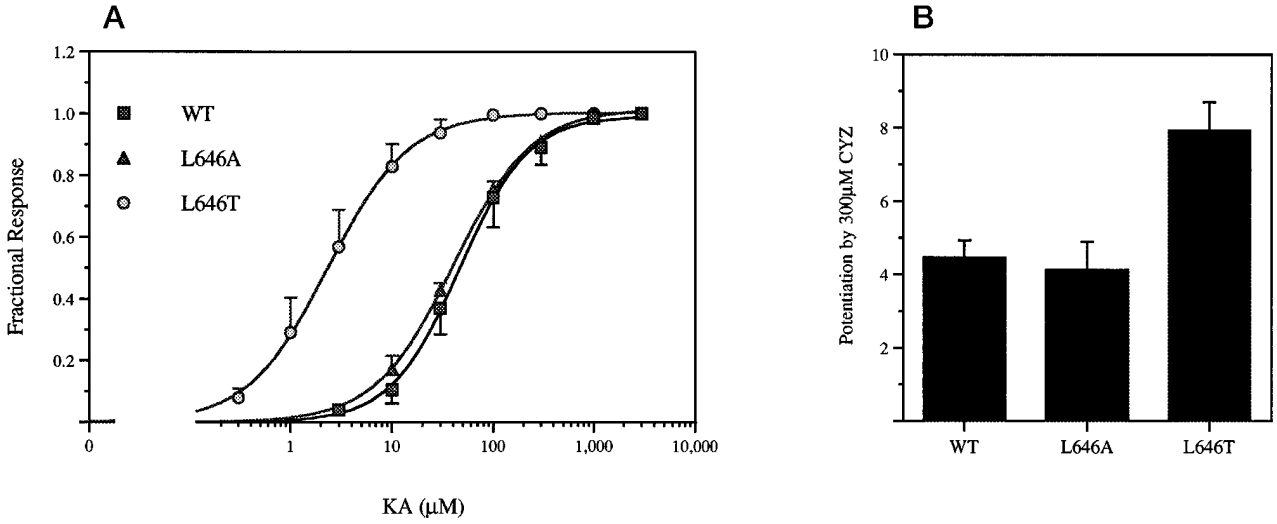

FIG. 3. **Effect of mutations at Leu-646 on KA-induced responses.** *A*, dose dependence of KA-induced currents of WT GluR1 (*rectangles*) and of the L646A (*triangles*) and L646T (*circles*) mutants. *Error bars* represent S.D. for groups of 6–11 oocytes. *B*, potentiation of 100 μM KA-induced responses by 300 μM CYZ (potentiation = (response to 100 μM KA with 300 μM CYZ)/(response to 100 μM KA without CYZ) − 1). *Error bars* represent S.E. In WT GluR1, responses to KA concentrations higher than 100 μM were not potentiated by 300 μM CYZ to a higher extent (data not shown), indicating that the increased receptor occupancy is not the basis for the higher extent of the CYZ-induced potentiation of the L646T mutant.

the relief of desensitization by CYZ, KA caused a stronger desensitization of the L646T GluR1 mutant channel than of the WT GluR1.

DISCUSSION

In the present study, we demonstrate that mutagenesis of amino acids in the GluR1 subunit of the AMPA receptor at locations corresponding to those of the ligand binding residues of LAOBP modifies the pattern of interactions of glutamatergic ligands. This observation supports the suggestion that the ligand binding domain of the AMPA receptors is similar to that of bacterial periplasmic proteins (16, 17) and is thus formed by two lobes harboring the ligand in the interlobe cleft.

The desensitizing agonists Glu and QA are shown here to interact with residues in both lobes. However, the two lobes do not have equal roles in their interaction with ligands. The consequences of a mutation in lobe I differ qualitatively from those of mutations in lobe II, although both types of mutations affect the ability of the desensitizing agonists Glu and QA to inhibit KA-induced responses. Indeed, although the mutation E398Q in lobe I increases the QA EC_{50} value (Fig. 2) and therefore reduces receptor occupancy, it does not modify the maximal extent of the QA-induced desensitization, while the mutations L646A and S650V in lobe II are found to significantly weaken the desensitizing action of QA. Accordingly, one may infer that the ligand interactions with lobe I control chan-

nel opening while the interactions with lobe II lead to desensitization. We do not exclude the possibility that lobe II is also involved in the process of channel activation in a transient closed lobe conformation, as a rightward shift in the apparent activation curves is observed for the S650V and L646A mutants. The proposition that lobe I is mainly involved in channel activation is also supported by the results of Li *et al.* (35), who by rapid application analysis observed that a lobe I mutation at Lys-445 caused a reduction in apparent affinity to AMPA and Glu without affecting the time course or extent of desensitization.

If indeed the above proposition is correct, one expects the weakly desensitizing agonist KA to interact mainly with residues in lobe I. Although we could not identify all the residues responsible for KA binding, our data from site-directed mutagenesis of GluR1 (this report) and of the chick kainate binding protein (33), a member of the Glu receptor family, suggest that at least the lobe I residues Glu-398, Tyr-401, and Tyr-446 are involved in KA binding and channel activation in GluR1. Our data on the lobe II mutant L646T also suggest that KA can be made to interact with lobe II. This interaction causes not only an increase of the apparent binding affinity but also, as we predicted, enhances the extent of KA-induced desensitization.

In this study we relate a change of conformation of the agonist binding domain to the process of channel desensitization. However, the latter change is clearly only one of the steps leading to the establishment of a desensitized close channel state. Indeed, the alternatively spliced flip/flop region of the protein was also shown to regulate desensitization, especially in heteromeric complexes (26, 36). However, the flip/flop region is probably not directly involved in the agonist binding step, since flip and flop receptors show similar pharmacological profiles. Moreover, the flip/flop region has been shown to be involved in the action of CYZ, an allosteric effector of AMPA receptors (34, 37).

The proposition that lobe I is involved in channel activation and the two lobes in desensitization implies the existence of two distinct receptor conformations, which is in line with the previous suggestion of the existence of two receptor states, one responsible for receptor activation and the other, exhibiting higher affinity, for desensitization (12). On the basis of the present results and in line with the venus flytrap mechanism that characterizes the LAOBP substrate binding dynamics, we propose that the transition of the GluR1 receptor channel from its resting state to an open state involves agonist binding mainly to lobe I, while additional interactions of the agonist with lobe II cause a closure of the two lobes, result in the trapping of the agonist, and permit the transition of the receptor channel to a desensitized state. Similar mechanisms are likely to be used by other Glu receptor channels since they all share a similarity of structure with the periplasmic binding proteins. Already, the ability of the *N*-methyl-D-aspartate subtype of glutamate receptors to trap agonists in an open blocked state of the channel has been documented (38). Thus, in spite of the large evolutionary distance separating bacteria from vertebrates, the bi-lobated structure of ligand binding domains and their function as a venus flytrap regulating ligand binding dynamics and membrane permeation has been conserved.

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