Mutant Cycle Analysis of the Active and Desensitized States of an AMPA Receptor Induced by Willardiines†

Pablo Kizelsztein,‡ Miriam Eisenstein,§ Nathalie Strutz,|| Michael Hollmann,|| and Vivian I. Teichberg*,‡

Departments of Neurobiology and Chemical Services, The Weizmann Institute of Science, Rehovot 76100, Israel, and the Laboratory of Glutamate Receptor Research, and The Max-Planck Institute for Experimental Medicine, Gottingen, Germany

Received April 27, 2000; Revised Manuscript Received July 10, 2000

ABSTRACT: The halogenated willardiines are agonists at the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptors. Although they differ only by the nature of the halogen substituent, they display marked differences in their efficacy to activate the receptor channel opening and in causing desensitization. We have studied the origin of the different agonist properties of the willardiines and in particular the nature of the structural element within the receptor binding domain that is able to distinguish between willardiines at a subatomic resolution of 0.6 Å (the difference in radius between F and Br) and allow (*S*)-5-fluorowillardiine to cause receptor desensitization much more than (*S*)-5-bromowillardiine. For this purpose, we analyzed, with the thermodynamic mutant cycle method, the active and desensitized states induced by the willardiines in the GluR1 subtype of AMPA receptors and GluR1 mutants in which residues E398, Y446, L646, and S650, within the agonist binding domain, were mutated. The results were used to generate a 3D model of the willardiine docking mode. We suggest that the active and desensitized states of the AMPA-R correspond, respectively, to the open-lobe and closed-lobe conformations of the agonist binding domain.

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)[1] subtype of glutamate receptors are multimeric integral membrane proteins (*1*), the four subunits (*2*, *3*) of which cooperate in the regulation of the cationic fluxes through the receptor channel. When exposed to glutamate (Glu) or to several other glutamatergic agonists, the AMPA receptor channels undergo two major conformational changes: the first is an activation step that causes the opening of the receptor channel and permits the flow of ions across the membrane; the second is a desensitization step that occurs a few milliseconds later and causes channel closure despite the continued presence of Glu (*4*). This process of desensitization is thought to shape the postsynaptic excitatory transmission because it limits the frequency at which the synaptic AMPA receptors can respond to Glu (*5*). Both the mechanisms of activation and desensitization are not fully understood in structural terms.

One particular unanswered question concerns the dependence of these processes on the nature of the agonist. All AMPA receptor agonists cause channel opening, but not all produce the same maximal activation or channel desensitization to the same extent.

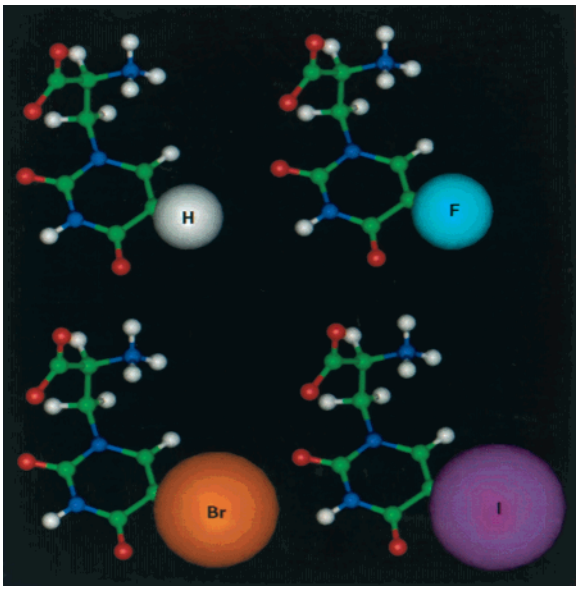

FIGURE 1: Structure of the (*S*)-5-Wlds showing the van der Waals volume of the substituent at the 5 position.

One striking example is provided by the (*S*)-willardiines (Wld). Those are a particularly interesting set of compounds because they have the very same basic chemical structure (Figure 1) but vary one from the other by the presence of a specific substituent at the 5 position. The H- and F-derivatives of Wld do not activate the AMPA receptor channel to the same maximal extent but are almost equally

† This research was supported by grants from the German-Israel Foundation for Scientific Research and Development (to M.H. and V.I.T.) and from the Minerva Foundation (to V.I.T.).

* To whom correspondence should be addressed. Telephone: 972-8-9342232; FAX: 972-8-9344131; E-mail: vivian.teichberg@weizmann.ac.il.

‡ Department of Neurobiology, The Weizmann Institute of Science.

§ Department of Chemical Services, The Weizmann Institute of Science.

|| Laboratory of Glutamate Receptor Research.

[1] Abbreviations: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA; glutamate, Glu; (*S*)-willardiine, Wld; (*S*)-5-fluorowillardiine, 5F-W; (*S*)-5-bromowillardiine, 5Br-W; (*S*)-5-iodowillardiine, 5I-W; GluR1 flop, $GluR1_o$; kainate, KA; cyclothiazide, CYZ; dose−response curve, DRC

10.1021/bi000962i CCC: $19.00 © 2000 American Chemical Society
Published on Web 09/28/2000

strong in causing receptor desensitization (*6*). In contrast, the Br- and I- derivatives are equipotent to (*S*)-5-fluorowillardiine (5F-W) as activating agonists but much weaker as desensitizing agonists (*6*). If, indeed, the size of the 5 substituent plays a major role in determining the extent of desensitization, then an intriguing question can be raised as to the nature of the element(s) in the receptor that can discriminate agonists at a subatomic dimension of 0.6 Å (the difference in van der Waals radii between F and Br) and cause, on that basis only, the receptor channel to undergo or not a change of conformation as significant as desensitization.

We have addressed this particular question and have analyzed in detail the interactions of four Wlds with the wild type (WT) GluR1 subtype of AMPA receptors and with GluR1 mutants in which the amino acids E398, Y446, L646, and S650, known to be present in the ligand binding domain (*7*, *8*), were mutated. To identify the GluR1 residues interacting specifically with the Wld 5 substituent, we used the thermodynamic mutant cycle method (*9*). This formalism provides values of the energetic coupling between moieties at protein−protein (*10*, *11*) or protein−ligand interacting interfaces (*12*, *13*). The halogenated Wld series provides a unique opportunity to analyze the functional consequences of ligand docking to AMPA receptors by double-mutant cycles because they can systematically be compared to their hydrogen counterpart and are relatively rigid. The results were interpreted in light of a three-dimensional (3D) model structure of the GluR1 ligand binding domain based on the recently established crystal structure of the soluble S1S2 GluR2 ligand binding domain (*8*).

EXPERIMENTAL PROCEDURES

Mutagenesis and GluR1$_{flop}$ Expression. The preparation of the GluR1 flop(GluR1$_o$) mutants used in this study, with the exception of E398AGluR1$_o$, was described (*7*). E398AGluR1$_o$ was prepared by PCR-mediated site-directed mutagenesis using mutagenic primers as described (*14*). Capped cRNA was transcribed using an Ambion MEGAscript kit.

Electrophysiological Recordings. Two electrode voltage clamp recordings from cRNA-injected Xenopus oocytes were performed as described (*7*). Agonists were applied by perfusion, and steady-state responses were measured following a 20-s exposure to the agonist. To establish the relative efficacies of the various agonists, all oocytes were exposed to test concentrations of a willardine and of kainate (KA) in the presence of 100 μM cyclothiazide (CYZ), and the current responses of KA were used as an internal control. When full dose−response curves (DRCs) for both a Wld and KA could not be established on the same oocyte, the ratios of the current responses were used for normalization. The maximal current response evoked by saturating concentrations of KA was set at a value of 1.0, and all other current responses were normalized accordingly. Currents were recorded on a chart recorder. Dose response curves were analyzed with Prism 3.0 software package (GraphPad Software, Inc). The various (*S*)-5-Wlds were purchased from Tocris. CYZ was purchased from RBI.

Propagation of Errors. Error propagation was calculated according to the following equation:

$$s_w = [(\partial w/\partial x)^2 s_x^{\,2} + (\partial w/\partial y)^2 s_y^{\,2}]^{1/2}$$

where *s* is the standard deviation, w is the resulting function, and x and y are the initial functions (*15*).

Molecular Modeling of GluR1 and the Docking of Wilardiines to the Binding Site. The three-dimensional (3D) model structure of the ligand binding domain of GluR1 was constructed on the basis of the experimental structure of the corresponding domain of GluR2 (*8*). The sequence of GluR1 is very similar to that of GluR2 showing 85.4% identity for the 253 amino acids comprising the ligand binding domain, with no inserts or deletions. An initial model of GluR1 was built using the homology module of MSI (MSI Inc., San Diego, CA). This model was energy-minimized with the Discover module, using the CVFF force field parameters. The Cα atoms were constrained to their initial positions during the minimization, which therefore served to relieve interatomic clashes, where they occurred, but the overall folding of the protein was not disrupted. The experimental structure of the GluR2 ligand binding domain includes a ligand—the kainate molecule (*8*). It is therefore assumed that the model structure of GluR1 derived from the GluR2 ligand binding domain is appropriate for ligand binding. Furthermore, any agonist of the GluR1 receptor is very likely to bind to the same site and maintain the conformation of the receptor/ligand complex. Therefore, we docked 5H-W into the binding site of GluR1 treating the latter essentially as a rigid body and allowing only very small local structural changes. Hence, the preferred position of 5H-W in the binding site of GluR1 was dictated by the mode of kainate binding in the GluR2 ligand binding domain as well as by the shape and chemical/physical characteristics of the GluR1 binding site and by the results of the point mutations in GluR1. Once the model-structure of the complex GluR1/5H-W was determined, we replaced the ligand with the substituted wilardiines, while maintaining the conformation of the binding domain.

RESULTS

The Activation by Wlds of WTGluR1 and GluR1 Mutants. We expressed the WTGluR1$_o$ homomeric receptor channel and the GluR1$_o$ mutants E398Q, Y446F, L646A, and S650A in frog oocytes. Using the two electrode voltage clamp technique, we performed dose−response curves (DRC) for the various Wlds to establish to what extent the mutations affected the potency of the Wlds (as measured by EC_{50} values) and their efficacy to open the receptor channel (as measured by the maximal current response). For efficacy comparisons, DRCs were carried out in parallel using kainate (KA) as an agonist because we previously found it to be equipotent toward both WTGluR1$_o$ and the above GluR1$_o$ mutants (*7*).

(*S*)-5-Willardiine (5H-W) and 5F-W produced small current responses in oocytes expressing the WTGluR1$_o$, and no DRCs could be established. Since the weakly desensitizing agonists (*S*)-5-bromowillardiine (5Br-W), (*S*)-5-iodowillardiine (5I-W), and KA did produce robust dose-dependent current responses, we surmised that the small current responses produced by 5H-W and 5F-W were due to their strongly desensitizing activity.

To support this notion and possibly reduce the occurrence of the desensitization process, all further DRCs were

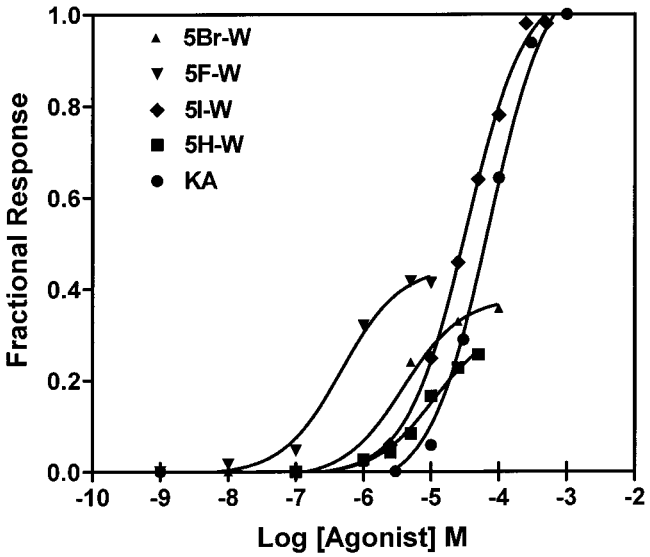

FIGURE 2: Dose-dependent current responses evoked by Wlds and KA from oocytes expressing WTGluR1$_o$. Each point on the figure corresponds to triplicate measurements performed on at least three oocytes. For sake of clarity, the standard deviations were not presented, but these did not exceed 15% of the response value presented.

established in the presence of 200 μM cyclothiazide (CYZ). This AMPA receptor allosteric effector was used because it is thought to diminish desensitization by stabilizing a nondesensitized agonist-bound closed state and improve thereby the transition to the open channel state (*16*). CYZ strongly potentiated the steady-state current responses evoked by the various agonists and as a result, DRCs could be obtained for 5H-W and 5F-W.

Figure 2 presents the DRCs for four Wlds and KA. The chloro derivative (*S*)-5-chlorowillardiine could not be studied because it is no more commercially available. The curves were normalized to the maximal current responses evoked by KA. CYZ did not significantly affect the EC_{50} values of 5Br-W and 5I-W (data not shown), as was already reported (*17*) for the effect of KA on GluR1$_o$.

It can be seen from the data presented in Figure 2 and in Table 1 that only 5I-W behaves similarly to KA as a full agonist of WTGluR1$_o$, whereas the other Wlds, even at the highest concentrations, fail to open the GluR1$_o$ receptor channel to its maximal current response. The partial agonism displayed by 5H-W has already been observed (*6*). 5H-W and its F- and Br- derivatives are thus found to act here as partial agonists with an order of efficacy I > F > Br > H. However, inspection in Table 1 of the EC_{50} values shows that these Wlds are more potent on WT GluR1$_o$ than KA and display an order of potency F > Br > H > I.

Figure 3 shows the DRCs obtained from oocytes expressing the GluR1$_o$ mutants E398Q, Y446F, L646A, and S650A, respectively. The Wlds are all more efficient agonists than KA in the activation of the E398Q and S650A GluR1 mutants and the order of efficacy is I > H > Br > F > KA while the order of potency is F > Br > I > H. The E398Q and S650A mutations seem to produce rather mild modifications of the protein as they affect the EC_{50} values by factors not exceeding 10-fold. There is however one exception for 5H-W since the EC_{50} value for its activation of E398QGluR1$_o$ is shifted to the right by a factor of 90.

Removing the contribution of the glutamate side chain by the E398A mutation causes, in comparison to the E398Q mutation, larger shifts to the right of the EC_{50} values by factors of 39, 31, and 51 for 5F-W, 5Br-W and 5H-W, respectively (data not shown). However, the EC_{50} values for 5I-W are not significantly affected either by the E398Q (Table 1) or E398A (data not shown) mutations.

In contrast to its effects on the E398Q and S650AGluR1$_o$ mutants, KA is the most efficient agonist for the activation of the Y446FGluR1$_o$ and L646AGluR1$_o$ mutants. The L646A mutation affects very significantly the potency of the Wlds and the EC_{50} values of 5F-W, 5Br-W, and 5H-W are shifted to the right by factors of 67, 244, and 247, respectively, in comparison to their values in the case of WTGluR1$_o$. Alhough the L646T mutation was found in a previous work to shift the KA EC_{50} value to the left by a factor of 10 (*2*), it was found here to shift to the right the EC_{50} values of 5F-W and 5Br-W by factors of 390 and 206, respectively (data not shown), suggesting a crucial role of the L646 residue in the interactions of the Wlds. The EC_{50} values of 5I-W and 5H-W for the activation of the L646TGluR1$_o$ mutant could not be determined because of the low solubility of these Wlds at high concentrations.

The Desensitization by Wlds of WTGluR1$_o$ and GluR1$_o$ Mutants. As mentioned above, the potentiating effects of CYZ have been attributed to its ability to increase the transition of the AMPA receptor channel to an active state as the latter becomes energetically more favorable than the desensitized state (*16*). Accordingly, the extent of potentiation by CYZ of the steady-state current response to a saturating dose of an agonist can be interpreted as an index of its desensitizing activity.

Table 2 presents the value of the CYZ potentiating effect calculated as the ratio F1/F2, where F1 is the current response in the presence of CYZ, and F2 is the response in its absence. To take into account the fact that the potentiation of the response depends on the concentration of CYZ, we expressed also a K_{pot} value calculated according to the formula $K_{pot} = [(F1/F2) - 1]/C$ where C is the concentration of CYZ. As shown below, K_{pot} is proportional to the ability of CYZ to reduce the concentration of receptors in the desensitized state. Table 2 includes as well the corresponding free energies calculated according to the relation $\Delta G = -RT \ln K_{pot}$.

Measuring the F1/F2 ratios for Glu and KA on WTGluR1$_o$, values of 20 ± 3.6 and 4.3 ± 0.7 were obtained, which are extremely close to those reported (*18*).

Inspection of the values in Table 2 suggests that indeed 5Br-W and 5I-W, which display K_{pot} values not in excess of $(3.6 \pm 0.4) \times 10^4$ and $(2.8 \pm 0.2) \times 10^4$, respectively, behave as weakly desensitizing agonists of both the WT and mutant GluR1$_o$. For comparison, KA, also considered as a weakly desensitizing agonist of GluR1$_o$ (*6*), displays a K_{pot} value of $(3.3 \pm 0.8) \times 10^4$. 5H-W and 5F-W, which are strongly desensitizing agonists, display large K_{pot} values with WTGluR1$_o$ but lower values with the GluR1$_o$ mutants.

Suggestive evidence that a decrease of K_{pot} values corresponds to a decrease of desensitization could be obtained on the basis of the properties of the E398GluR1$_o$ mutant since DRCs with robust current responses could be established for the activation of this mutant (in contrast to WTGluR1$_o$; see above) by both 5F-W and 5H-W, in the absence of CYZ (data not shown). Interestingly, the E398Q mutation abolishes

Table 1: Efficacy, Potency, and Free Energy of Interaction of Willardiines with Wild Type GluR1 and Mutants[a]

	agonist	WT GluR1$_o$	E398QGluR1$_o$	Y446FGluR1$_o$	L646AGluR1$_o$	S650AGluR1$_o$
H-W	potency EC_{50} (μM)	11.5 ± 1.83	1014 ± 201	116 ± 22	2842 ± 234	56 ± 4.5
	efficacy	0.26 ± 0.02	0.82 ± 0.05	0.44 ± 0.08	0.3 ± 0.02	0.63 ± 0.02
	ΔGEC_{50} (kcal/mol)	6.71 ± 0.09	4.07 ± 0.11	5.35 ± 0.11	3.46 ± 0.05	5.78 ± 0.05
F-W	potency EC_{50} (μM)	0.47 ± 0.08	4.57 ± 0.19	4.3 ± 0.79	31.5 ± 0.5	0.87 ± 0.24
	efficacy	0.42 ± 0.04	0.41 ± 0.01	0.4 ± 0.07	0.34 ± 0.06	0.4 ± 0.01
	ΔGEC_{50} (kcal/mol)	8.60 ± 0.10	7.26 ± 0.02	7.29 ± 0.10	6.12 ± 0.01	8.24 ± 0.16
Br-W	potency EC_{50} (μM)	2.83 ± 0.39	10.2 ± 1.93	43.3 ± 3.8	691 ± 109	13.6 ± 1.82
	efficacy	0.36 ± 0.03	0.71 ± 0.01	0.36 ± 0.08	0.22 ± 0.06	0.5 ± 0.1
	ΔGEC_{50} (kcal/mol)	7.54 ± 0.08	6.78 ± 0.11	5.93 ± 0.05	4.29 ± 0.09	6.61 ± 0.08
I-W	potency EC_{50} (μM)	33.6 ± 6.5	14.2 ± 0.7	121 ± 10	59.6 ± 6.92	67 ± 9
	efficacy	0.98 ± 0.01	1.00	0.54 ± 0.07	0.32 ± 0.03	1.00
	ΔGEC_{50} (kcal/mol)	6.08 ± 0.11	6.39 ± 0.03	5.32 ± 0.05	5.74 ± 0.07	5.67 ± 0.08
KA	potency EC_{50} (μM)	80 ± 5	90 ± 7	100 ± 15	83 ± 11	78 ± 12
	efficacy	1.00	0.37 ± 0.02	1.00	1.00	0.38 ± 0.04

[a] EC_{50} values and maximal current responses were derived from analysis of at least three dose−response curves per agonist carried out on different oocytes. Values are mean ± SD. ΔGEC_{50} calculated according to the relation: $\Delta GEC_{50} = -RT \ln EC_{50}$.

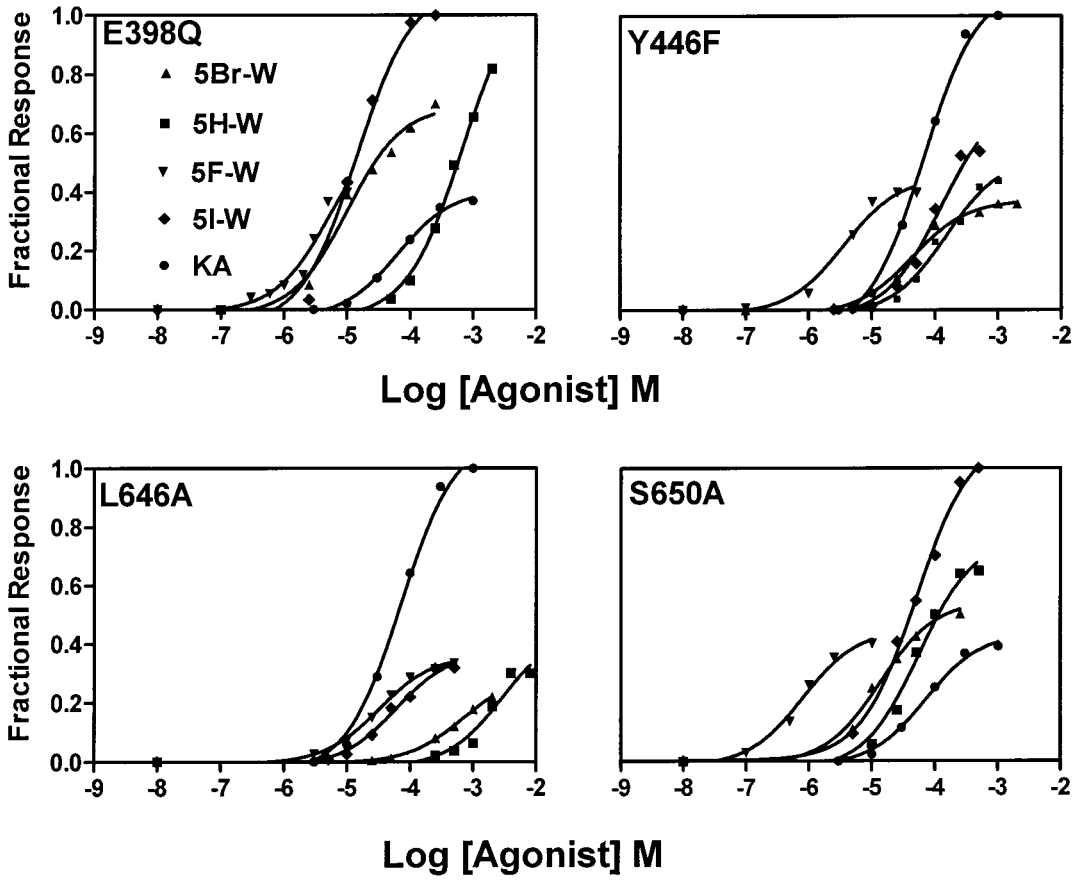

FIGURE 3: Dose-dependent current responses evoked by Wlds and KA from oocytes expressing mutant GluR1$_o$. All agonist responses were recorded in the presence of 200 μM CYZ. For each mutant, the current responses evoked by saturating concentrations of an agonist were normalized to the value of the maximal current response. For reference, the average responses ± SEM of the various GluR1$_o$ mutants to the test concentration of 200 μM KA + 200 μM CYZ were as follows: WTGluR1$_o$: 783 ± 127 nA (number of oocytes n = 14); E398QGluR1$_o$: 333 ± 60 nA (n = 9); L646AGluR1$_o$: 720 ± 134 nA (n = 15); Y446FGluR1$_o$: 1163 ± 201 nA (n = 8); S650AGluR1$_o$: 233 ± 45 nA (n = 7). Each point on the figure corresponds to triplicate measurements performed on at least three oocytes. For sake of clarity, the standard deviations were not presented, but these did not exceed 15% of the response value presented. The values of the Hill coefficient varied between 1.1 and 1.9. These variations can be accounted for by the K phenotype of the mutations produced (*21*)

almost completely the 5F-W induced desensitization but produced a lesser effect on 5H-W.

Calculating the Coupling Energies of the Wld 5-Halogen Moiety. Intermolecular interactions between a ligand and a receptor can be analyzed using double mutant cycles as illustrated in Figure 4 for the case of 5F-W and the L646AGluR1$_o$ mutant. This particular cycle comprises a "mutant" of 5F-W, i.e., 5H-W where the F moiety is "substituted" with a H moiety. The coupling energy between the F moiety of the ligand and the L646 side chain is given by the relation:

$$\Delta G_{cpl} = [\Delta G(\text{WT/5F-W}) - \Delta G(\text{WT/5H-W})] - [\Delta G(\text{L646A/5F-W}) - \Delta G(\text{L646A/5H-W})]$$

where the four ΔG values correspond to the interactions

Table 2: Potentiation by Cyclothiazide of the Current Responses to Willardiines of WT GluR1$_0$ and Mutants[a]

	agonist	5H-W	5F-W	5Br-W	5I-W
WT GluR1$_0$	F1/F2	28 ± 2	26 ± 4	4.6 ± 0.4	3.5 ± 0.1
	K_{pot} (× 10^4)	27 ± 2	25 ± 4	3.6 ± 0.4	2.5 ± 0.1
	ΔG (kcal/mol)	−7.38 ± 0.4	−7.34 ± 0.09	−6.19 ± 0.06	−5.98 ± 0.02
E398Q GluR1$_0$	F1/F2	9 ± 1	3 ± 0.2	1.4 ± 0.1	1.8 ± 0.1
	K_{pot} (× 10^4)	8 ± 1	2 ± 0.2	0.4 ± 0.1	0.8 ± 0.1
	ΔG (kcal/mol)	−6.66 ± 0.07	−5.84 ± 0.06	−4.89 ± 0.15	−5.3 ± 0.07
Y446F GluR1$_0$	F1/F2	6 ± 0.7	6.1 ± 0.3	1.5 ± 0.3	1.6 ± 0.04
	K_{pot} (× 10^4)	5 ± 0.7	5.1 ± 0.3	0.5 ± 0.3	0.6 ± 0.04
	ΔG (kcal/mol)	−6.39 ± 0.08	−6.4 ± 0.03	−5.03 ± 0.35	5.13 ± 0.04
L646A GluR1$_0$	F1/F2	21 ± 3	4.3 ± 0.6	2.4 ± 0.3	3.8 ± 0.2
	K_{pot} (× 10^4)	20 ± 3	3.3 ± 0.6	1.4 ± 0.3	2.8 ± 0.2
	ΔG (kcal/mol)	−7.2 ± 0.09	−6.14 ± 0.1	−5.63 ± 0.12	−6.04 ± 0.04
S650A GluR1$_0$	F1/F2	18 ± 0.4	10.4 ± 0.3	4.1 ± 0.1	2.5 ± 0.2
	K_{pot} (× 10^4)	17 ± 0.4	9.4 ± 0.3	3.1 ± 0.1	1.5 ± 0.2
	ΔG (kcal/mol)	−7.11 ± 0.01	−6.76 ± 0.02	−6.1 ± 0.02	−5.68 ± 0.08

[a] F1/F2 values ± SEM ($n > 10$) were obtained by measuring the extent of potentiation by 100 μM CYZ of the steady-state current response to a saturating dose of a given agonist. Similar sets of data were obtained using 200 μM CYZ indicating that CYZ was present at saturation. K_{pot} was calculated as described in the text.The free energy $\Delta G = -RT \ln K_{pot}$.

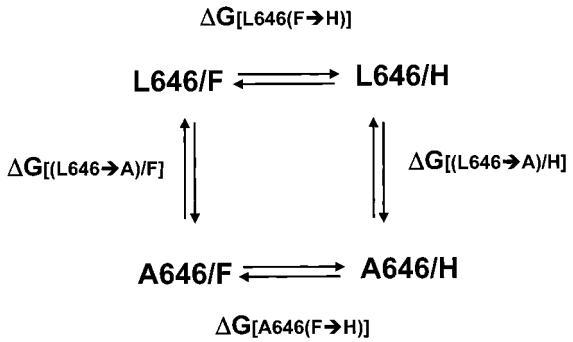

FIGURE 4: Double-mutant cycle to analyze the pairwise interaction for the case of 5F-W and the L646AGluR1$_o$ mutant. The resulting coupling energy corresponds to the interaction between L646 and the F-moiety.

Table 3: Coupling Energies for the Interactions between the Wld Halogen Moiety and Residues in GluR1$_0$ Active State and Desensitized State[a]

moiety		E398	Y446	L646	S650
F-	Δ_{Act}	−1.30 ± 0.17	−0.05 ± 0.2	−0.77 ± 0.14	−0.57 ± 0.21
	Δ_{Des}	0.52 ± 0.21	0.10 ± 0.23	−0.25 ± 0.22	0.26 ± 0.23
Br-	Δ_{Act}	−1.88 ± 0.19	0.25 ± 0.17	0.0 ± 0.16	0.0 ± 0.15
	Δ_{Des}	1.30 ± 0.26	−0.42 ± 0.39	−0.38 ± 0.21	0.19 ± 0.17
I-	Δ_{Act}	−3.15 ± 0.18	−0.60 ± 0.19	−2.91 ± 0.18	−0.52 ± 0.17
	Δ_{Des}	3.19 ± 0.20	0.74 ± 0.24	3.15 ± 0.20	0.49 ± 0.19

[a] Values in kcal/mol are presented as mean ± propagated error. The EC_{50} values used for the calculations of the coupling energies (Δ_{act}) were taken from Table 1. ΔGcpl(K_{pot}) was calculated using the ΔG values presented in Table 2, and ΔDes was calculated from ΔDes = ΔGcpl(K_{pot}) − ΔAct.

between the binding site wild type or mutant residue with the wld fluoro or hydrogen moieties, respectively.

The coupling energies ΔG_{act} presented in Table 3 were calculated for the interactions between the halogen at the 5 position of the Wld and amino acid residues in the ligand binding domain of GluR1 in its active conformation (values derived from EC_{50} values). To determine the parallel coupling energies in the desensitized state ΔG_{Des}, we made use of the K_{pot} values. The biological meaning of the K_{pot} values is not intuitive but can be reached by the following analysis:

K_{pot} is derived from measurements at equilibrium of agonist current responses in the presence (F1) or absence of CYZ (F2). Assuming that F1 and F2 are proportional to the fraction of receptors in the active state in the presence and absence of CYZ, respectively, eqs 1 and 2 will express F1 and F2 on the basis of the following equilibria:

(a) In the presence of CYZ:

$$[C] + [R] \overset{K1}{\longleftrightarrow} [CR]$$

$$[CR] + [A] \overset{K2}{\longleftrightarrow} [CRA]$$

$$[R] + [A] \overset{K3}{\longleftrightarrow} [RA]$$

$$[CRA] \overset{K4}{\longleftrightarrow} [CR_DA]$$

$$[RA] \overset{K5}{\longleftrightarrow} [R_DA]$$

$$F1 = ([RA] + [CRA])/[R_{tot}] \quad (1)$$

(b) In the absence of CYZ:

$$[R] + [A] \overset{K3}{\longleftrightarrow} [RA]$$

$$[RA] \overset{K5}{\longleftrightarrow} [R_DA]$$

$$F2 = [RA]/[R_{tot}] \quad (2)$$

where [R] is the concentration of free receptor in the resting state, [C] is the CYZ concentration, [A] is the agonist concentration, [R_D] is the receptor in its desensitized conformation, and [R_{tot}] is the total concentration of receptor. By substituting [RA] for $k3$[R][A] in eqs 1 and 2 and [RCA] by $k1k2$[R][C][A] in eq 1, one obtains that F1 = [R][A]($k3$ + $k1k2C$)/[R_{tot}] and F2 = [R][A]$k3/R_{tot}$ and thus that

$$F1/F2 = 1 + (k1k2/k3)[C] \quad (3)$$

A rearrangement of eq 3 gives

$$[F1/F2 - 1]/[C] = K_{pot}$$

where $K_{pot} = k1k2/k3$. By substituting the respective values of $k1$, $k2$, and $k3$, one can show that

$$K_{pot} = [C][RA]/[CRA]$$

and thus that K_{pot} represents the equilibrium constant of CYZ to the receptor in its active conformation.

An understanding of the biological significance of the coupling energy derived from double mutant cycles using K_{pot} values can be reached by presenting a double-mutant cycle involving the ternary complex between RA and C and using the property of additivity of thermodynamic mutant cycles (*19*).

$$\begin{array}{ccccccccc} & & k2 & & k5 & & k7 & & k10 & \\ R' + A + C & \leftrightarrow & R'A + C & \leftrightarrow & \mathbf{CR'A} & \leftrightarrow & \mathbf{CR'A'} & \leftrightarrow & C + R'A' & \leftrightarrow & C + R' + A' \\ \updownarrow k1 & & \updownarrow k4 & & k_a\updownarrow & & k_b\updownarrow & & \updownarrow k9 & & \updownarrow k1 \\ R + A + C & \leftrightarrow & RA + C & \leftrightarrow & \mathbf{CRA} & \leftrightarrow & \mathbf{CRA'} & \leftrightarrow & C + RA' & \leftrightarrow & C + R + A' \\ & & k3 & & k6 & & k8 & & k11 & & \end{array}$$

A′ and R′ stand for mutant ligand and mutant receptor, respectively. Since the coupling constant K_{cpl} is defined for the cycle in bold as $K_{cpl(Kpot)} = k_b/k_a$, one can readily see, by applying the principle of free energy conservation, that

$$K_{cpl(Kpot)} = (k10k7k6k3)/(k2k5k8k11)$$

By deriving the coupling energy $\Delta G_{cpl(Kpot)} = -RT \ln K_{cpl(Kpot)}$, then

$$\Delta G_{cpl(Kpot)} = -RT \ln(k10k3/k11k2) - RT \ln(k6k7/k5k8)$$

As $-RT \ln(k10k3/k11k2)$ represents the coupling energy, in the absence of CYZ, between the receptor residue (mutated in R′) and the halogen moiety of the agonist (substituted in A′), while $-RT \ln(k6k7/k5k8)$ represents the coupling energy, in the presence of CYZ, between the receptor residue and the agonist halogen moiety, one can write:

$$\Delta G_{cpl(Kpot)} = \Delta G_{Des} + \Delta G_{Act}$$

where ΔG_{Des} corresponds to the coupling energy of the agonist with the receptor in the absence of CYZ, i.e., in its desensitized conformation, while ΔG_{Act} corresponds to the coupling energy of the agonist with the receptor in the presence of CYZ, i.e., in its active conformation and thus, $\Delta G_{Act} = \Delta G_{cpl(EC50)}$. Accordingly,

$$\Delta G_{Des} = \Delta G_{cpl(Kpot)} - \Delta G_{cpl(EC50)}$$

Table 3 shows the calculated coupling energies ΔG_{Des} and ΔG_{Act} characterizing the interactions of the willardiine halogens with residues in the ligand binding domain either in its desensitized or active conformation. Negative values are indicative of favorable/stable interactions between the interacting moieties, whereas positive values indicate destabilizing interactions. $\Delta G_{cpl} = 0$ corresponds to the situation when the effects of the mutations are additive, i.e., independent of each other. This occurs when the mutated moieties are noninteracting. For interpretation purposes, only ΔG_{cpl} values clearly above 1 kcal/mol were considered.

Inspection of Table 3 shows that the interactions of the Wld halogen substituents with the E398 residue are all favorable when the receptor is in its active conformation and unfavorable in the desensitized conformation. Moreover, there seems to be a dependence on the halogen size since the strength of the interactions shows the order I > Br > F. The coupling energies for the halogen moieties with Y446 and S650 are of limited amplitude suggesting a lack of interaction either in the active or in the desensitized conformation. The iodine moiety forms favorable interactions with L646 and E398 in GluR1 active conformation but very unfavorable interactions in the desensitized conformation.

DISCUSSION

The AMPA receptor, as other ligand-gated receptor channels, undergoes two major changes in conformation upon agonist binding. The first is a transition from a resting state to an active state (or states) that allows with high probability the opening of an integral ion channel; the second is a transition to a desensitized state (or states) characterized by high affinity agonist binding and very low probability of channel opening.

Significant differences in the desensitization process have been observed for the GluRs in the flip or flop versions, and the allosteric effector CYZ shows much larger potentiation effects on $GluR1_{flip}$ than on $GluR1_o$ (*16*, *17*). CYZ blocks the fast desensitization step that takes place after activation by Glu of $GluR1_{flip}$ and only slows down the desensitization of $GluR1_o$. However, the important and relevant observations in the present context is that CYZ potentiates the equilibrium responses of both $GluR1_{flip}$ and $GluR1_o$ and that the extent of potentiation depends on the nature of the agonist. When studied in oocytes, the equilibrium responses of $GluR1_{flip}$ and $GluR1_o$ measured in the presence of CYZ are in fact only quantitatively but not qualitatively different. Thus, the conclusions reached in the present work using $GluR1_o$ are unlikely to be affected by the nature of the flip/flop domain.

Although extremely similar in structure, 5H-W and 5F-W display a much larger desensitizing action on neuronal AMPA-Rs than 5Br-W and 5I-W (*6*). In a comparison of the steady-state current responses, the following order of agonist efficacy 5I-W > 5Br-W > 5F-W > 5H-W was found (*6*). Thus, the stronger the desensitization, the lower was the efficacy. A similar trend is observed here on $GluR1_o$-expressing oocytes since 5H-W and 5F-W elicit much smaller steady-state current responses than 5Br-W and 5I-W, and the presence of CYZ markedly increases their magnitude.

An intuitive interpretation of the K_{pot} values (but see the results section) is that they reflect the specific desensitizing activity of an agonist since large values are obtained for agonists such as 5H-W and 5F-W which in the absence of CYZ evoke only very small current responses from $GluR1_o$, whereas small values are obtained with agonists such as 5Br-W and 5I-W which, in the absence of CYZ, evoke large responses.

CYZ was reported to slow rather than prevent the desensitization of $GluR1_o$ (*17*), yet it is found here to increase the steady-state current responses of all agonists. To account for these findings, one may suggest that $GluR1_o$ has two desensitized states differing from each other by their binding affinity to CYZ. Drawing an analogy with the nicotinic acetylcholine receptor which is postulated to exist in four conformational states, a resting state B, an active-open state A, and two desensitized states I (an initial desensitized state) and D (a final desensitized state) (*20*), one could propose that CYZ slows down the transition of $GluR1_o$ to the inactive state I but abolishes the transition to the D state. Accordingly, one would expect the agonist-mediated steady-state current responses to be enhanced by CYZ as full desensitization would not occur. Using the same analogy and applying the

Monod Wyman Changeux (MWC) model of allosteric proteins to GluR1, one may also explain the differential efficacy of agonists (as observed when applied at saturating concentrations and in the presence of CYZ; Figures 2 and 3) as the binding of agonists to the resting state will decrease the pool of receptors present in the active-open state (*21*).

One of the expected properties of a receptor channel operating according to the MWC model is however the occurrence of spontaneous channel openings resulting from the postulated agonist-independent equilibrium between the resting and the active states. Such openings have not been observed yet for any AMPA-R but have been found to occur in NMDA-R, another member of the family of ionotropic Glu receptors (*22*). As it stands, one can possibly account for the differential agonist efficacy displayed by the Wlds, by their interactions to one or more inactive states of $GluR1_o$, but one cannot decide yet whether the latter correspond(s) to an additional desensitized state or alternatively to the resting state or to both.

It is of interest to note that neither the K_{pot} values on their own nor the orders of potency or efficacy that the various Wlds display for the different $GluR1_o$ mutants show a clear relation with one of the physicochemical properties of the 5 substituent such as size, hydrophobicity, or electron-withdrawing capacity. This is not unexpected because one ought to realize that by measuring the effects of a mutation of a given amino acid present in the ligand binding domain, one may possibly deduce its role in ligand binding but not the details of the ligand binding mode such as the ligand orientation within the binding domain.

One way to approach the question of the ligand binding mode and improve the resolution of the analysis is to use the double-mutant cycle method that allows the determination of the nature and strength of the interactions taking place at the interface between interacting proteins (*11*) or between a protein and its ligand (*12*). In the present case, we assumed that all Wlds have a similar docking mode, i.e., that the configuration at the carbon α of the Wld and the links of its bonded amino and carboxyl groups with specific amino acid residues in the ligand binding domain are invariant. We further assumed that the different properties of the Wlds as GluR1 agonists are mainly due to the varying contributions of the physicochemical characteristics of the Wld 5 substituent to its interactions with ligand binding domain residues. To remove one at a time each of these interactions without adding new interactions or significantly perturb the structure of the ligand binding domain, we systematically compared the effects of a 5-halogenated Wld to that of a reference 5H-Wld while mutating ligand binding residues either to alanine or to a close analogue. Thus, L646 and S650 were mutated into alanine, Y446 into phenylalanine, and E398 into glutamine. These amino acids were chosen on the basis of previous work (*7*) indicating that these residues play crucial roles in the activation and desensitization of $GluR1_o$.

The use of "not-to-alanine" mutations in double mutant cycles is considered to be problematic because such mutations may add new interactions rather than remove the interaction under study (*23*). However, the E398Q mutation produces much milder effects on the EC_{50} values of the various Wlds than the E398A mutation. Thus, in the present case, the E398Q mutation that removes the charge of the side chain but not its hydrogen bonding capacity or its space-filling property seems to be more appropriate than the more drastic E398A mutation. A similar argument can be made for the Y446F mutation that removes only a hydroxyl moiety without affecting the aromaticity.

The coupling energies that result from the application of double mutant cycles refer to the energy of interactions between the halogen substituent and the residue occupying the mutated position in WTGluR1. Two different thermodynamic parameters were fed in the mutant cycles.

The first thermodynamic parameter is derived from the agonist potency value, i.e., the EC_{50}. The second thermodynamic parameter is the free energy $\Delta G_{cpl(Kpot)}$ derived from K_{pot} values. As seen in Results, $\Delta G_{cpl(Kpot)}$ is composed of two terms ΔG_{Des} and $\Delta G_{cpl(EC50)}$,which correspond respectively to the coupling energies of the agonist with the receptor, in the absence or presence of CYZ. Since the agonist in the absence of CYZ tends to stabilize the receptor desensitized conformation, the coupling energy ΔG_{Des} corresponds to the interaction energy between the agonist and the desensitized conformation of the receptor. ΔG_{Des} is not measurable directly but is derived from the measurements of $\Delta G_{cpl(Kpot)}$ and $\Delta G_{cpl(Ec50)}$ according to the relation: $\Delta G_{Des} = \Delta G_{cpl(Kpot)} - \Delta G_{cpl(EC50)}$ (see Results).

Inspection of Table 3 reveals that the small F moiety has smaller coupling energies than the large iodine moiety. Moreover, the coupling energies are of opposite signs in the active and desensitized states. In the active state, the large iodine moiety forms favorable and more stable interactions with residues E398 and L646 than the smaller Br and F moieties. In the desensitized state, the iodine moiety interactions are all very unfavorable. Apparently, the desensitized state of $GluR1_o$ accommodates well Wlds with small but not large size halogen substituents while the opposite situation holds for the active state that accommodates well Wlds with large size halogen substituents. On that basis, one may possibly conclude that the active state corresponds to an open-lobe conformation of the ligand binding domain while the desensitized state would correspond to a close-lobe conformation.

It should be noted that the absolute changes in coupling energy for the halogen interactions with E398 in the desensitized or active state (i.e., $|\Delta G_{Des} + \Delta G_{Act}|$) are up to 1.82 ± 0.27 kcal/mol for F, 3.18 ± 0.32 kcal/mol for Br, and 6.34 ± 0.26 kcal/mol for I. This suggests that the change in conformation that the receptor undergoes from the active to the desensitized state is affected much more by the interactions of the large I atom than by those of the small F atom. This suggestion would be in line with the venus flytrap model of desensitization of GluR1 (*7*) which proposed that the ligand binding domain closes or entraps the ligand upon desensitization. This closure might not readily occur with ligands such as 5Br-W and 5I-W.

One may try to interpret the above results in relation to the recently established 3D structure of the ligand binding domain of $GluR2_o$ in its liganded form (*8*). This structure was established using KA as a ligand, and although the latter is often considered as a non- or weakly desensitizing agonist, the desensitization it produces is probably underestimated (*24*). Thus, the 3D structure of the KA binding core is likely to correspond to a close or desensitized conformation of the binding domain since this is the thermodynamically preferred conformation of the receptor−ligand complex at equilibrium.

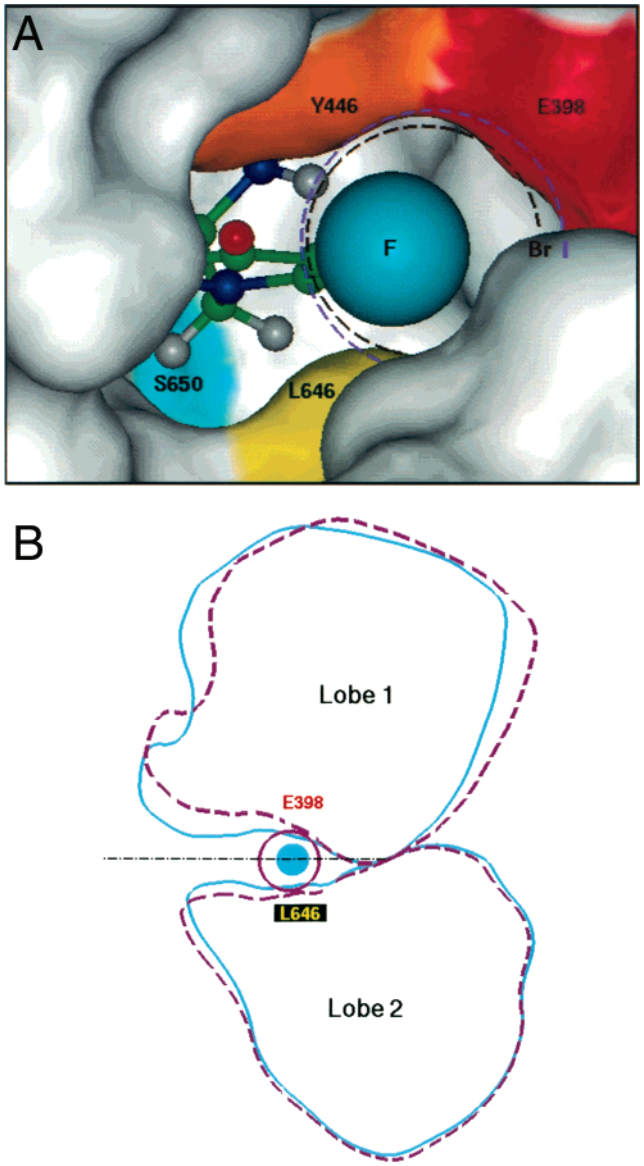

FIGURE 5: (A) Space-filling model of the docking of the Wlds in the ligand binding cavity of GluR1. This cavity has the same dimensions as in the 3D structure of GluR2 S1S2 in its KA-liganded form. 5F-W fits nicely and the cavity dimensions are clearly compatible with the accommodation of the F moiety. The circles around the F atom show the dimensions of the Br and I atoms, respectively. The I moiety cannot be fitted within the cavity because of a steric hindrance by E398, Y446, and L646. (B) Plausible model of the ligand binding domain in its active and desensitized states. The active conformation (in purple) has a wider cleft by at least 0.8 Å than the desensitized conformation (in blue) and allows the binding of 5I-W.

Figure 5, panel A presents part of the ligand binding domain of GluR1 modeled by homology with the structure of the soluble S1S2 $GluR2_o$ (*8*). It shows the cavity in which the ligand binds, and its walls are color-coded by the residues that were mutated. The Wlds were docked assuming that the location of the Cα atom and the links formed by its bonded amino and carboxyl groups with specific amino acid residues in the ligand binding domain are identical to those formed by KA in the soluble S1S2 $GluR2_o$. Wlds are rigid molecules that can adopt two main conformations related by rotation about the CH2-ring bond. In the two conformers, the halogen points either toward the carboxyl group or the amino group. We chose the latter conformation because the I- moiety of 5-IW was found to interact favorably with residues L646 and E398 in the receptor active conformation, and we assume that the conformation of this agonist does not change upon receptor desensitization.

The cavity accommodates very nicely the desensitizing 5H-W and 5F-W. 5H-W forms electrostatic interactions with R481, S650, T476, and P474 and van der Waals contacts, through the aromatic ring, with Y446, L646, and with the CH_2 groups of E398, displacing the water molecule found near the latter in the experimental structure. Substitution of H in the 5 position by F does not change significantly the size of the molecule and thus 5F-W can be easily accommodated in the cavity. However, the latter cannot accommodate the bulkier and "nondesensitizing" 5Br-W and 5I-W, although they harbor atoms that are larger than F by only 0.6 and 0.8 Å, respectively. Clashes, in particular with E398, are noticed. The conformation of E398 cannot be changed so as to relieve the clash, and energy minimization at this point changes the conformation of the receptor. Such a conformation change results in a more open structure, where the bromine and iodine moieties can be accommodated. Thus, the open structure accepts all the halogens while the "closed" accepts only the hydrogen and fluorine Wld substituents.

As expected from the values of the coupling energies, the halogen moieties are all far from S650. Our observation that a large atom such as I interacts favorably with both lobe 1 residues E398 and the lobe 2 residue L646 during the ligand-induced channel activation step (i.e., opening of the channel) but very unfavorably in the desensitized conformation, suggests that the active conformation of the ligand binding domain has a wider cavity than that of the desensitized conformation (Figure 5, panel B). The difference of cavity width should be at least 0.8 Å at a critical location, to accommodate the I- moiety. The change in conformation taking place upon desensitization is thus expected to be a relative closure of the cavity. It may also involve a rotation of one lobe with respect to the other as typically observed in periplasmic binding proteins upon ligand binding (*25*).

If a closure of the ligand binding cavity of GluR1 is a prerequisite for desensitization, i.e., for ion channel closure, then agonists that do not fit into a closed ligand binding cavity will not produce desensitization. Thus, the present work offers a possible answer to the question of why agonists strongly desensitize the receptor while others do not. More specifically, it may explain why only a 0.6−0.8 Å difference in van der Waals radii between F and Br/I allows 5F-W to desensitize the receptor while 5Br-W and 5I-W do so to a much lesser extent. The main differences between the active and the desensitized state would thus be the degree of opening of the ligand binding cavity and the stability of interaction with the ligand.

An important issue that remains to be addressed is the nature of the desensitization stimulus, i.e., does the agonist induce the desensitization process, or does it stabilize a preexisting desensitized conformation?

ACKNOWLEDGMENT

We are extremely indebted to Dr. A. Horovitz for most constructive comments and extensive discussions. We thank Drs. S. Edelstein, P. Leff, and B. Attali for fruitful advice.

V.I.T. holds the Louis and Florence Katz-Cohen professorial chair of neuropharmacology.

REFERENCES

1. Hollmann M (1999) in *Ionotropic Glutamate Receptors in the CNS* (Jonas P., Monyer H., Eds.), pp 3−98, Springer, Berlin.
2. Mano, I., and Teichberg, V. (1998) *Neuroreport 9*, 327−331.
3. Rosenmund, C., Stern-Bach, Y., and Stevens, C. F. (1998) *Science 280*, 1596−1599.
4. Trussell, L. O., and Fischbach, G. D. (1989) *Neuron 3*, 209−218.
5. Jones, M. V., and Westbrook, G. L. (1996) *Trends Neurosci. 19*, 96−101.
6. Patneau, D. K., Mayer, M. L., Jane, D. E., and Watkins, J. C. (1992) *J. Neurosci. 12*, 595−606.
7. Mano, I., Lamed, I., and Teichberg, V. I. (1996) *J. Biol. Chem. 271*, 15299−15302.
8. Armstrong, N., Sun, Y., Chen, G., and Gouaux, E. (1998) *Nature 395*, 913−917.
9. Carter, P. J., Winter, G., Wilkinson, A. J., and Fersht, A. R. (1984) *Cell 38*, 835−840.
10. Horovitz, A., Serrano, L., Avron, B., Bycroft, M., and Fersht, A. R. (1990) *J. Mol. Biol. 216*, 1031−1044.
11. Horovitz, A. (1996) *Fold. Des. 1*, R121−R126.
12. Steyaert, J., Haikal, A. F., and Wyns, L. (1994) *Proteins 18*, 318−323.
13. Hidalgo, P., and MacKinnon, R. (1995) *Science 268*, 307−310.
14. Hollmann, M., Maron, C., and Heinemann, S. (1994) *Neuron 13*, 1331−1343.
15. Parratt, L. G. (1971) *Probability and Experimental Errors in Science*, Dover Publications Inc., New York.
16. Partin, K. M., Fleck, M. W., and Mayer, M. L. (1996) *J. Neurosci. 16*, 6634−6647.
17. Partin, K. M., Patneau, D. K., and Mayer, M. L. (1994) *Mol. Pharmacol. 46*, 129−138.
18. Partin, K. M., Patneau, D. K., Winters, C. A., Mayer, M. L., Buonanno, A. (1993) *Neuron 11*, 1069−1082.
19. Horovitz, A. (1987) *J. Mol. Biol. 196*, 733−735.
20. Edelstein, S. J., and Changeux, J. P. (1998) *Adv. Protein Chem. 51*, 121−184.
21. Leff, P. (1995) *Trends Pharmacol. Sci. 16*, 89−97.
22. Turecek, R., Vlachova, V., and Vyklicky, L. J. (1997) *Eur. J. Neurosci. 9*, 1999−2008.
23. Faiman, G. A., and Horovitz, A. (1996) *Protein Eng. 9*, 315−316.
24. Stern-Bach, Y., Russo, S., Neuman, M., and Rosenmund, C. (1998) *Neuron 21*, 907−918.
25. Sun, Y. J., Rose, J., Wang, B. C., and Rsiao, C. D. (1998) *J. Mol. Biol. 278*, 219−229.

BI000962I