

Conformational Changes in the Ligand-binding Domain of a Functional Ionotropic Glutamate Receptor*[§]

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Fluorescence resonance energy transfer was used to determine the structural changes in the extracellular ligand-binding segment in a functional glutamate receptor that contains the ligand-binding, transmembrane, and C-terminal segments. These studies indicate that the structural changes previously reported for the isolated ligand-binding domain due to the binding of partial and full agonists are also observed in this functional receptor, thus validating the detailed structure-function relationships that have been previously developed based on the structure of the isolated ligand-binding domain. Additionally, these studies provide the first evidence that there are no significant changes in the extent of cleft closure between the activated and desensitized states of the glutamate bound form of the receptor consistent with the previous functional investigations, which suggest that desensitization is mediated primarily by changes in the interactions between subunits composing the receptor.

Ionotropic glutamate receptors are the main excitatory neurotransmitter receptors in the mammalian central nervous system and hence play a key role in processes such as learning and memory (1–5). Glutamate binding to the extracellular soluble ligand-binding domain (S1S2)¹ segment of the protein triggers a series of conformational changes that leads to receptor activation (formation of a cation selective transmembrane channel) and subsequent desensitization. The determination of the structure of the S1S2 for the GluR2 subunit of the glutamate receptor (6–9) complemented with spectroscopic investigations

(10–13), in combination with the vast existing electrophysiological data on the native receptor (9, 14, 15), have provided the first direct structural insight into how the changes at the ligand-binding site lead to the sequence of conformational changes that regulate ion flow in this class of important proteins. The validity of these inferences hinges on the assumption that the isolated ligand-binding domain is a good model for the domain in a functional receptor, *i.e.* the ligand-binding domain in the presence of the functional unit, namely the transmembrane segments forming the ion channel. The assumption that the S1S2 protein is a representative model of the domain in the full glutamate receptor is currently based on equilibrium ligand binding assays that suggest similar ligand binding properties for the two proteins (16, 17) and recent UV-visible spectroscopic investigations that indicate a similar electronic environment for the antagonist 6-cyano-7-nitro-2,3-dihydroxyquinoxaline in the S1S2 protein as in the full-length receptors expressed in cells (18). In this report we have used fluorescence resonance energy transfer (FRET) to provide the first direct confirmation that the ligand-binding domain of a functional glutamate receptor exhibits the same conformational changes (cleft closure) due to agonist binding as that observed in the structures of the S1S2 protein. For these FRET investigations we have modified the cysteine light Δ N-GluR4 homomeric receptors. The Δ N-GluR4 receptors have the extracellular N-terminal domain (residues 1–402) deleted from the GluR4 flip subunit of the α -amino-5-methyl-3-hydroxy-4-isoxazole propionate subtype of the glutamate receptor and represent the minimum model construct essential for the full function, namely agonist-induced activation and desensitization, of the receptor. The two extracellular non-disulfide-bonded cysteine residues (426 and 529) in this Δ N-GluR4 construct were mutated to serine, providing a receptor with no accessible cysteines. Green fluorescent protein (GFP) was then introduced at the N terminus to this modified protein to serve as the acceptor, and cysteines were introduced at specific positions and tagged using a maleimide derivative of terbium chelate to serve as the donor molecule and distances between donor and acceptor measured based on the FRET efficiency.

MATERIALS AND METHODS

Mutations and eGFP-tagged Cysteine Light GluR4 Construct Preparation—Using the construct for the GluR4-flip receptor with the first 402 residues deleted (generously provided by Dr. Keinänen, University of Helsinki, Helsinki, Finland) the two accessible non-disulfide-bonded cysteine residues, Cys⁴²⁶ present in the extracellular side as well as Cys⁵²⁹ present in the first transmembrane segment of the receptor, were mutated to serine using the Stratagene QuikChange site-directed mutagenesis kit (Stratagene). For introducing the GFP at the N terminus of this double mutant, the Δ N-GluR4-C426S-C529S construct was amplified with PCR using primers containing XhoI and BamHI restriction enzyme sites; the PCR product was digested by using the XhoI and BamHI restriction enzymes and subcloned into the corresponding sites of pEGFP-C1 plasmid (Invitrogen). This construct was digested with NheI and BamHI, and the 2.3-kb fragment, which contained the Δ N-GluR4-C426S-C529S construct cDNA fused with coding sequence for eGFP at the N terminus, was cloned into pcDNA3.1. The construct thus obtained (Δ N*-GFP) served as the background construct on which residues 444, 446, 653, and 686 were then individually mutated to cysteine to yield the four constructs used for the FRET study. For the donor only experiments the same mutations were performed on the Δ N-GluR4-C426S-C529S without the presence of GFP tag (Δ N*). Mutations in the plasmids were introduced using the Stratagene QuikChange site-directed mutagenesis kit (Stratagene, CA), and the integrity of the final

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¹ The abbreviations used are: S1S2, soluble ligand-binding domain; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; eGFP, enhanced GFP.

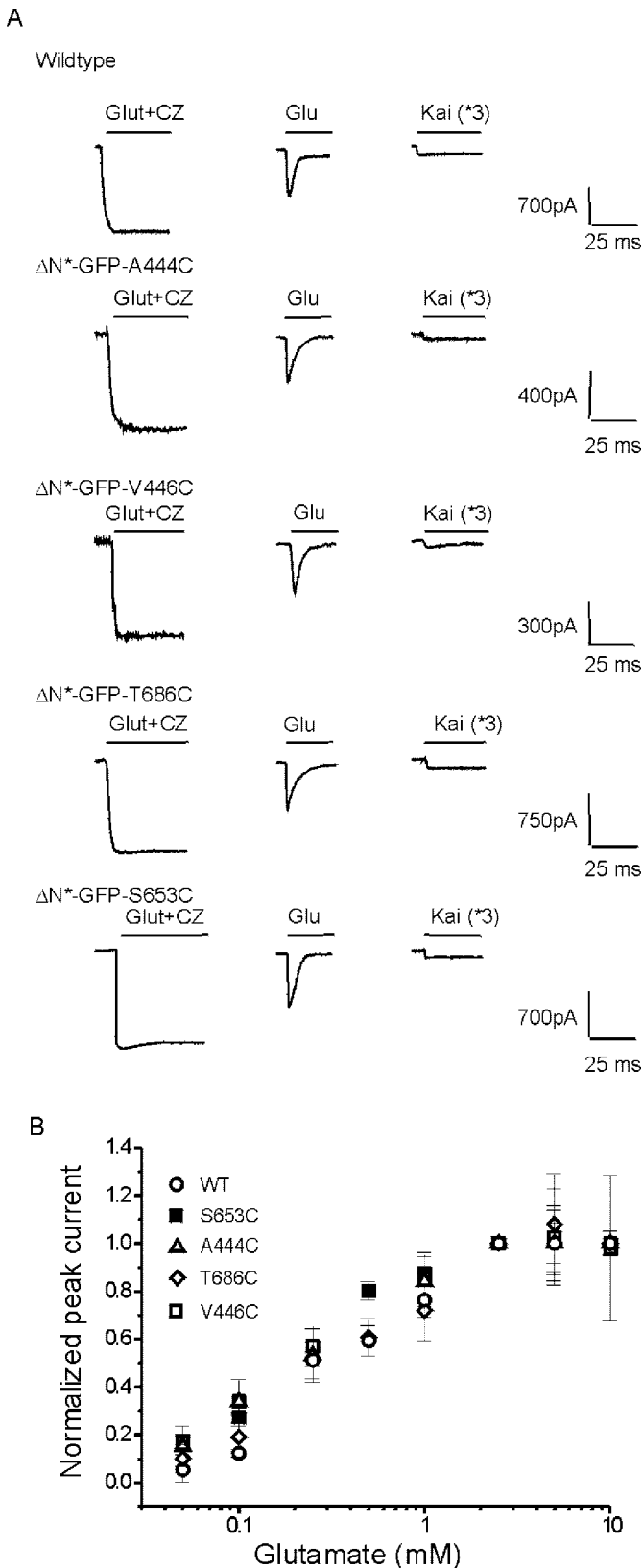


FIG. 1. *A*, whole-cell currents evoked from HEK-293 cells transiently transfected with the plasmid for wild-type or ΔN^* -GFP mutant receptors tagged with the terbium chelate due to application of (i) 10 mM glutamate in the presence of 100 μ M cyclothiazide, (ii) 10 mM glutamate, and (iii) 10 mM kainate. The duration for which the ligand solution was applied is shown above each current trace. *B*, dependence of the peak whole-cell currents on the glutamate concentration. Each data point is an average of at least three measurements from three cells. The whole-cell currents from different cells were normalized to the current obtained at 2.5 mM glutamate.

TABLE I
Lifetimes for the donor only (τ_D in μ s), sensitized emission lifetimes (τ_{DA} in μ s), and the distances (r_{DA} in \AA) computed based on the lifetimes for the various sites probed

Site	Apo		Kainate bound		Glutamate bound		Glutamate bound in presence of CZ					
	Lifetime		Lifetime		Lifetime		Lifetime					
	τ_D	τ_{DA}	τ_D	τ_{DA}	τ_D	τ_{DA}	τ_D	τ_{DA}				
S653C	1049	47.4	36	1101	27.9	32	1089	12.4	28	1080	13.6	28
T686C	1059	42.1	35	1101	28.8	33	1110	23.4	31	1074	22.1	31
A444C	940	13.0	29	935	14.1	29	931	13.8	29	947	12.8	29
V446C	939	13.9	29	940	13.8	29	935	12.9	29	951	13.2	29

constructs verified by sequencing the coding region for both strands of the DNA.

Human Embryonic Kidney 293 (HEK-293) Cell Cultures—HEK-293 cells (ATCC CRL 1573) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM glutamine, 50 μ g/ml penicillin, and 50 μ g/ml streptomycin in 250-cc culture flasks. Transfections were performed using FuGENE 6 (Roche Applied Science) or Lipofectamine (Invitrogen) transfection reagent with 0.5 μ g of modified glutamate receptor cDNAs per ml of medium. Transfected cells were allowed to grow for 1–3 days before use.

Receptors expressed in HEK-293 cells were washed in extracellular buffer and labeled at specific cysteine sites with the maleimide derivative of terbium chelate for an hour. The cells were then washed with extracellular buffer until no free dye was observed in the wash and were subsequently used for fluorescence lifetime measurements. For electrophysiological characterization, the HEK-293 cells expressing the mutant receptors were labeled with the probe for half an hour then rinsed once with extracellular buffer prior to the measurements.

Electrophysiological Measurements—For whole cell current recordings, transfected HEK-293 cells were voltage-clamped at a holding potential of -60 mV, and solutions were applied using a home mode U tube with a 100- μ m aperture. The electrode solution, for the electrophysiological measurements, contained 140 mM CsCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, 2 mM Na₂ATP, and 10 mM HEPES (pH 7.4); the extracellular bath solution contained 145 mM NaCl, 1.8 mM MgCl₂, 1 mM CaCl₂, 3 mM KCl, 10 mM glucose, and 10 mM HEPES (pH 7.4). Currents were amplified with an Axon 200B amplifier (Axon Instruments, Foster City, CA), low pass filtered at 1 kHz. The filtered signal was digitized using a Labmaster DMA digitizing board controlled by Axon PClamp software. All the experiments were performed at room temperature and at pH 7.4.

Fluorescence Measurements—The fluorescence measurements were obtained using a TimeMaster™ model TM-3/2003 (Photon Technologies Inc.), a cuvette-based fluorescence lifetime spectrometer. A nitrogen/dye laser system provided high intensity illumination that was fiber-optically coupled to the sample compartment, which contains a thermostatable cuvette holder with microstirrer. Emitted light was collected by quartz optics at 90 degrees to the incident beam and passed through a 200-mm monochromator to a stroboscopic detector. Data were collected using Felix 3.0 software and analyzed using Kaleidagraph (Synergy software).

Determination of the R_0 Value for GFP and Terbium Tagged to the Glutamate Receptor—The overlap integral J was calculated using Equation 1,

$$J = \frac{\sum_i F_D(\lambda_i) \cdot \epsilon_A(\lambda_i) \cdot \lambda_i^4}{\sum_i F_D(\lambda_i)} \quad (\text{Eq. 1})$$

where $F_D(\lambda)$ is the fluorescence spectrum of terbium, $\epsilon_A(\lambda)$ the absorption spectrum of GFP tagged to the glutamate receptor, and λ is the wavelength. The calculations were performed using a spreadsheet (Microsoft Excel), and the value of J was determined to be $2.82 \cdot 10^{15} \text{ m}^{-1} \text{ cm}^{-1} \text{ nm}^4$. Using this value for J , R_0 was then calculated using Equation 2,

$$R_0^6 = \frac{8.785 \cdot 10^{-5} \cdot \kappa^2 \cdot \phi_D \cdot J}{n^4} \quad (\text{Eq. 2})$$

where κ is the orientation factor, ϕ is the quantum yield of terbium, and n is the refractive index. Using 2/3 as the value for κ (19), 0.6 for ϕ

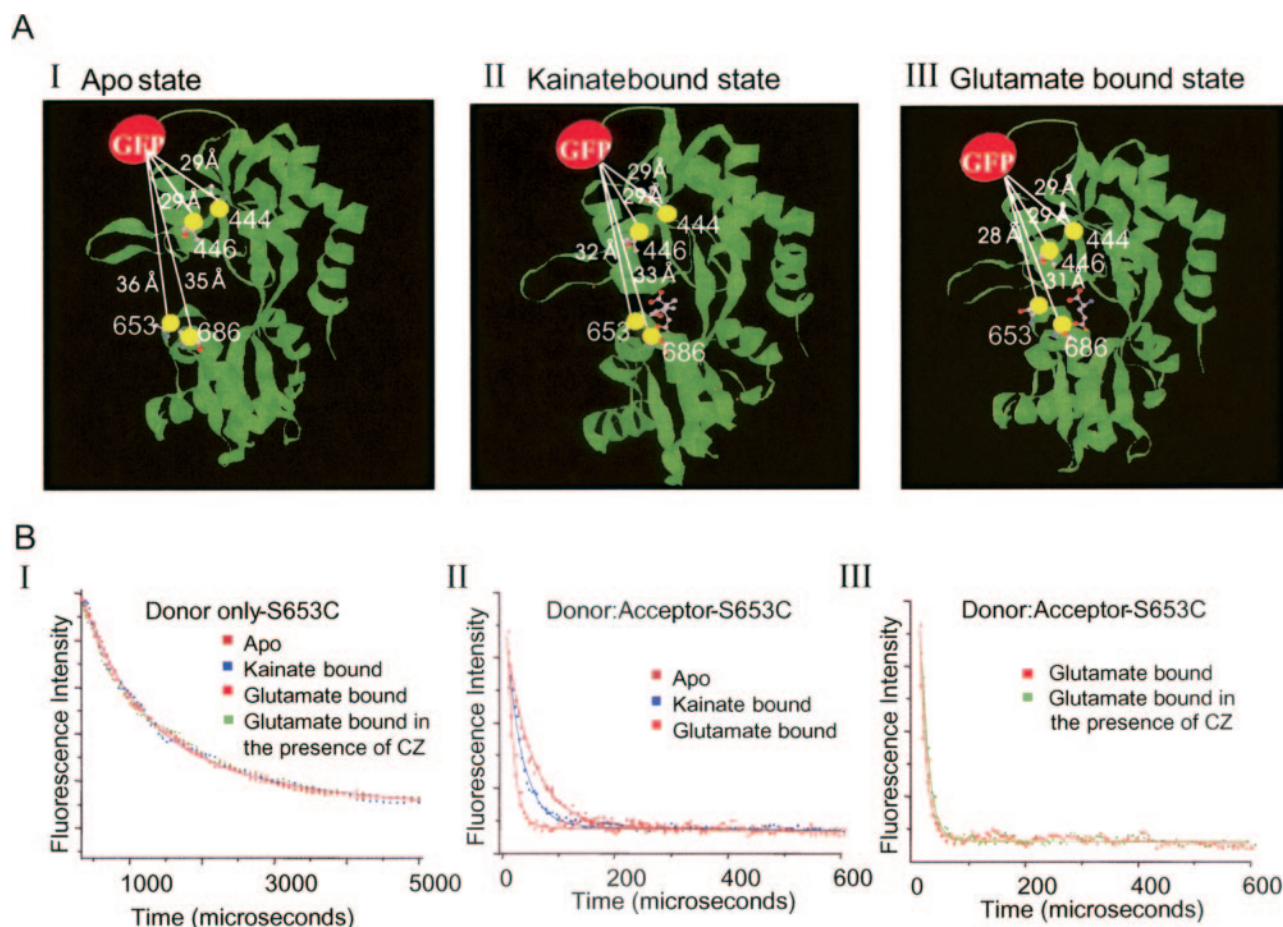


FIG. 2. *A*, the x-ray structure of the ligand-binding domain is used to show the sites that were labeled in the present study. The distances in the apo, kainate bound, and glutamate bound forms between the various residues and GFP as determined by the FRET measurements are shown in the structures (I to III), respectively. *B*, excited state lifetimes when donor is attached at site 653. *I*, lifetime for donor only protein as measured at 488 nm; *II*, sensitized emission of GFP as measured at 511 nm in the apo, kainate bound, and glutamate bound forms; and *III* sensitized emission of GFP for the glutamate bound forms in the presence and absence of cyclothiazide.

(Clontech), and 1.3 as the value for n , R_0 was determined to be 59 Å. Since the absorption spectrum of terbium was not significantly altered for the different sites probed, this R_0 value was used for determining the distances for all the sites probed.

Distance Calculations Based on Lifetime—The distance between the donor:acceptor fluorophores was determined by measuring the time constants of donor fluorescence decay in the absence (τ_D) and sensitized emission of the acceptor due to energy transfer from the donor (τ_{DA}) and using Equation 3 given by Förster's theory of singlet-singlet energy transfer.

$$R = R_0 \left(\frac{\tau_{DA}}{\tau_D - \tau_{DA}} \right)^{1/6} \quad (\text{Eq. 3})$$

The error in the distances determined by FRET assuming a 2/3 as the value of κ was established by measuring the fluorescence anisotropy, p , of the donor and acceptor. A detailed calculation outlining the conversion of the p values of the donor and acceptor in terms of the error in FRET is provided by Haas *et al.* (19). The value of p was determined by measuring the intensity of the light in the parallel (I_{\parallel}) and perpendicular (I_{\perp}) positions.

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (\text{Eq. 4})$$

Using Equation 4, the value of p was determined to be 0.47 for GFP tagged to glutamate, and this was not significantly altered by the presence of terbium chelate at the different sites on the protein. Since the terbium chelate is unpolarized it does not contribute to the error. Therefore using the value of 0.47 for GFP, the maximum error in the FRET distances was determined to be +12% to -9% of the actual distances using the calculations of Haas *et al.* (19).

RESULTS AND DISCUSSION

Functional Characterization of the Mutant Proteins—Each of the mutant proteins with the donor and acceptor tags were individually characterized using electrophysiological measurements to ensure that they exhibited similar activation and desensitization as the wild type receptor. Whole cell currents were obtained in the presence of partial agonist kainate (10 mM) and full agonist glutamate (10 mM) under desensitizing conditions (absence of cyclothiazide) and non-desensitizing conditions (presence of cyclothiazide (100 μ M)) (Fig. 1A). These studies show that the whole cell currents are similar for the wild type and mutant proteins tagged with fluorophores with 1) the activation of full agonist glutamate leading to significantly larger currents than the activation by partial agonist kainate, 2) the receptors being predominantly desensitized in the continued presence full agonist glutamate with similar lifetimes for desensitization (9–14 ms), and 3) desensitization being blocked in the presence of cyclothiazide. Additionally, the dose-response curves as determined by the dependence of the peak currents due to activation by various concentrations of glutamate are not significantly different for the wild type and mutant proteins tagged with fluorophores (Fig. 1B) indicating that the equilibrium binding properties are not significantly different. Since the fluorescence resonance energy transfer experiments probe the equilibrium end states these mutants are excellent models for this study.

Fluorescence Resonance Energy Transfer—The distances between the reference GFP protein tagged at the N terminus and various residues in the S1 and S2 segments tagged with the terbium chelate donor were determined in the apo and various ligated states by using the lifetimes of acceptor-sensitized emission and donor emission without an acceptor (Table I; Fig. 2 and supplemental material). When sites 444 and 446 are tagged with the terbium chelate fluorophore, no changes are observed in the sensitized emission lifetime between apo, kainate bound, glutamate bound, and glutamate bound in the presence of cyclothiazide (Table I; Fig. 2 and supplemental material). This indicates that the distance between the donor and acceptor is the same in all these states of the protein. Since residues 444 and 446 are present in domain 1 of the glutamate receptor (Fig. 2), it can be concluded that the position of the acceptor GFP with respect to domain 1 is the same for the protein in the apo, kainate bound, glutamate bound, and glutamate bound in the presence of cyclothiazide.

The lifetime for the sensitized emission when the donor is attached to residues 653 or 686, on the other hand, is state-dependent and follows the order apo state > kainate bound > glutamate bound ~ glutamate bound in the presence of cyclothiazide (Fig. 2). This order indicates that distance between acceptor GFP and donor tagged at these sites decreases upon going from the apo state to the agonists bound states and also that full agonist glutamate induces a larger decrease in the distance relative to partial agonist kainate. Furthermore, the absence of an affect on the lifetime due to cyclothiazide suggests that the distance between the donor and acceptor is not altered significantly by the presence of cyclothiazide. Residues 653 and 686 are present on domain 2 of the ligand-binding segment (Fig. 2); therefore the movement of these residues toward GFP upon binding agonists with larger decrease in distance upon binding glutamate relative to kainate is in complete agreement with the x-ray structures of the isolated ligand-binding domain which indicate partial cleft closure upon binding kainate and full cleft closure upon binding glutamate.

These results provide conclusive proof that the structural changes in the isolated ligand-binding domain are replicated in a fully functional glutamate receptor. In addition, the present results also provide the first evidence that there is no significant change in the degree of cleft closure between the open and desensitized states of the protein (glutamate bound in the presence of cyclothiazide and glutamate bound states). The lack of significant changes within a domain upon going from the activated to desensitized state is consistent with the current hypothesis based on structural and functional investigations, where it has been suggested that cleft closure predominantly mediates channel opening (activation), while desensitization is predominantly mediated by the decoupling of the interface between the S1S2 dimers (7, 20, 21).

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