Luminescence Resonance Energy Transfer Investigation of Conformational Changes in the Ligand Binding Domain of a Kainate Receptor*[^s]

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The apo state structure of the isolated ligand binding domain of the GluR6 subunit and the conformational changes induced by agonist binding to this protein have been investigated by luminescence resonance energy transfer (LRET) measurements. The LRET-based distances show that agonist binding induces cleft closure, and the extent of cleft closure is proportional to the extent of activation over a wide range of activations, thus establishing that the cleft closure conformational change is one of the mechanisms by which the agonist mediates receptor activation. The LRET distances also provide insight into the apo state structure, for which there is currently no crystal structure available. The distance change between the glutamate-bound state and the apo state is similar to that observed between the glutamate-bound and antagonist UBP-310-bound form of the GluR5 ligand binding domain, indicating that the cleft for the apo state of the GluR6 ligand binding domain should be similar to the UBP-310-bound form of GluR5. This observation implies that the apo state of GluR6 undergoes a cleft closure of 29–30° upon binding full agonists, one of the largest observed in the glutamate receptor family.

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[^2]: The abbreviations used are: AMPA, α-amino-5-methyl-3-hydroxy-4-isoxazole propionate; ATPO, 2-amino-3-[5-tert-buty1-3-(phosphonooxy)-4-isoxazolyl]propionic acid (ATPO)-bound state and the 2-amino-2-carboxethyl-3-(2-carboxyphene-3-ylmethyl)-5-methylpyrimidine-2,4-dione (UBP-310)- and (S)-1-(2-amino-2-carboxethyl)-3-(2-carboxybenzyl) pyrimidine-2,4-dione (UBP-302)-bound states (18, 33). It is therefore still unknown which of these structures most closely represents the apo state of the protein.
were expressed, purified, and characterized as detailed previously (13). Briefly, the protein was expressed in *Escherichia coli* Origami-B (DE3) cells, and first purified by a nickel-nitrilotriacetic acid HiTrap affinity column (GE Healthcare) followed by thrombin digestion overnight to remove the histidine tag. Anion exchange chromatography (Q-Sepharose Fast Flow from GE Healthcare) was then performed to remove the thrombin. The functionality of all purified proteins was established by saturation binding of $[^3H]kainate$ to the proteins (supplemental Fig. 1).

**Labeling and LRET Measurements**—0.5 μM protein in phosphate-buffered saline with 1 mM glutamate was labeled with a 1:1 ratio of the maleimide derivatives of fluorescein (Biotium, Hayward, CA) and triethylengediaminehexaacetic acid chelate of terbium (TTHA-Tb) (Invitrogen) or with terbium chelate alone for donor:acceptor and donor-only proteins, respectively. The labeled protein was diazylated extensively against phosphate-buffered saline and used for the LRET measurements.

Fluorescence measurements were obtained using a *TimeMaster™* model TM-3/2003 (Photon Technology International, Inc., Birmingham, NJ) lifetime spectrometer. The fluorescence data were obtained with at least two different protein samples, and for each sample the data were an average of five shots (hence the data shown is an average of 10 data points). The data from each independent sample were also fit to ensure no significant differences between samples of the same protein. The distances were calculated from the donor-only and donor:acceptor lifetimes using Förster’s theory for energy transfer, and the $R_0$ values were determined for each of the constructs by obtaining the fluorescence and absorption spectrum of donor and acceptor, respectively, tagged to the proteins (16). The error reported for the distances has been determined using error propagation due to the error in the lifetimes.

**Electrophysiological Measurements**—For the whole cell current recordings, human embryonic kidney 293 cells were transfected with wild type or T661E mutant GluR6-flip receptors. The transfected cells were voltage-clamped at a holding potential of −60 mV, and solutions were applied using a homemade U-tube mixing device that had a 100-μm aperture. The electrode solution, for the electrophysiological measurements, contained 140 mM CsCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM EGTA, 2 mM Na$_2$ATP, and 10 mM HEPES (pH 7.4); the extracellular bath solution contained 145 mM NaCl, 1.8 mM MgCl$_2$, 1 mM CaCl$_2$, 3 mM KCl, 10 mM glucose, and 10 mM HEPES (pH 7.4). Currents were amplified with an Axon 200B amplifier (Molecular Devices, Sunnyvale, 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.

**RESULTS AND DISCUSSION**

**Conformational Changes Induced upon Ligand Binding**—The LRET lifetimes as measured by the sensitized emission of the acceptor fluorescein due to luminescence transfer from the donor terbium chelate for the wild type and T661E mutant proteins are shown in Fig. 2 and listed in Table 1. There are currently two structures, the kainate-bound and glutamate-
Conformational Changes of Kainate Receptor

FIGURE 2. Fluorescence lifetimes. The LRET lifetime as measured by the sensitized emission of the acceptor in the GluR6 ligand binding domain labeled with fluorescein and Ttha-Tb in the apo (black) and kainate- (red), domoate- (blue), glutamate- (green), and methyl glutamate-bound (magenta) states for the wild type S398C-S666C (A) and wild type S398C-S689C (B); and in the apo (black), kainate- (red) and glutamate-bound (green) states for S398C-S666C-T661E (C) and S398C-S689C-T661E (D).

To obtain a comprehensive correlation between the extent of cleft closure and activation, a wide range of activations was investigated using the wild type and T661E mutant and agonists kainate, domoate, methyl glutamate, and glutamate (supplemental Fig. 2). The extent of activation was determined from the maximal whole cell currents mediated by saturating concentrations of the various agonists normalized to the currents mediated by saturating concentrations of glutamate. It should be noted that the normalized activation does not represent the channel open probability. Hence although the normalized activation is 1 for glutamate, the normalized open probability for glutamate activating the GluR6(Q)-flip receptors has been previously reported to be 0.96 (35). The correlation between the changes in the LRET-based distances between residue 398, in domain 1, and residues 666 and 689, in domain 2, for the different ligands bound to the wild type and T661E mutant and activation is shown in Fig. 3. These results show that the distances between residue 398 and residues 666 and 689 exhibit a negative correlation to the extent of activation over the full range, i.e. the full agonists exhibit shorter distances and partial agonists exhibit longer distances between the sets of residues. Because residue 398 is in domain 1 and residues 666 and 689 are in domain 2 of the isolated ligand binding domain, the decrease in distance between the two domains is consistent with a cleft closure conformational change. Thus, it can be concluded that agonists with greater activations induce larger cleft closure, and cleft closure could be the coupling mechanism by which the agonist mediates channel opening over the full range of activations.

Apo State of the Protein—The LRET-based distances indicate that the apo state is more open relative to the agonist-bound states, with a 3.1- and 5.7-Å increase in distance between residues 398 and residues 666 and 689, respectively, upon going from the glutamate-bound form to the apo state. This result is consistent with a cleft closure conformational change induced by agonist binding.

Because there are no apo state or antagonist-bound structures available for the GluR6 ligand binding domain, the distance change between the apo state and glutamate-bound state for GluR6 is compared with the changes between the three antagonist-bound structures and the glutamate-bound structure for the closely related ligand binding domain of the GluR5 subunit (18, 33). It should be noted that the glutamate-bound forms of the GluR6 and GluR5 ligand binding domains exhibit similar distances between residue 398 and residues 666 and 689, thus allowing for such a comparison (18, 33).

The three antagonist-bound structures of GluR5 have slightly varying degrees of open clefts relative to the full agonist glutamate-bound state, with the cleft being 28–30° more open.

### TABLE 1

Lifetimes for donor-only (D) and donor:acceptor (DA) tagged GluR6 ligand binding domain constructs and distances determined based on these lifetimes

| Protein              | Ligated state | \( \tau_D \) (µs) | \( \tau_{DA} \) (µs) | Distance (Å) |
|----------------------|---------------|-----------------|-----------------|--------------|
| S398C-S666C          | Apo           | 1699 ± 27       | 192 ± 2         | 42.5 ± 0.7   |
|                      | Glutamate     | 1745 ± 32       | 130 ± 3         | 39.4 ± 0.9   |
|                      | Kainate       | 1753 ± 34       | 149 ± 4         | 40.3 ± 1     |
|                      | Domoate       | 1712 ± 30       | 144 ± 3         | 40.3 ± 1     |
|                      | Methyl glutamate | 1675 ± 27     | 129 ± 3         | 39.6 ± 0.9   |
| S398C-S689C          | Apo           | 1745 ± 24       | 185 ± 2         | 42.0 ± 0.6   |
|                      | Glutamate     | 1774 ± 12       | 84 ± 2          | 36.3 ± 0.9   |
|                      | Kainate       | 1718 ± 23       | 97 ± 2          | 37.5 ± 0.8   |
|                      | Domoate       | 1684 ± 24       | 96 ± 2          | 37.5 ± 0.8   |
|                      | Methyl glutamate | 1721 ± 22     | 75 ± 2          | 35.8 ± 1     |
| S398C-S666C-T661E    | Apo           | 1786 ± 24       | 212 ± 3         | 42.9 ± 0.6   |
|                      | Glutamate     | 1733 ± 32       | 135 ± 3         | 39.7 ± 0.9   |
|                      | Kainate       | 1777 ± 25       | 174 ± 3         | 41.4 ± 0.6   |
|                      | Domoate       | 1711 ± 30       | 176 ± 2         | 41.8 ± 0.7   |
|                      | Glutamate     | 1698 ± 34       | 66 ± 1          | 35.1 ± 0.7   |
|                      | Kainate       | 1690 ± 31       | 98 ± 2          | 37.7 ± 0.8   |

bound forms, available for the GluR6 ligand binding domain (13). The changes in distance calculated from the LRET lifetimes between the kainate- and glutamate-bound states for residues 398–666 and 398–689 are 1 and 1.5 Å, respectively, for both sets of residues, similar to the changes of 0.9 and 1.2 Å observed between the kainate and glutamate crystal structures (13). This agreement in distance changes establishes that these two x-ray structures are a good representation of the average dynamic structure of the protein in buffer.

To obtain a comprehensive correlation between the extent of cleft closure and activation, a wide range of activations was investigated using the wild type and T661E mutant and agonists...
in the ATPO, UBP-310, and UBP-302 antagonist-bound states (18, 33). Apart from the small changes in the extent of cleft opening, the axis of rotation in the ATPO structure is also different from that in the UBP-310- and UBP-302-bound states (Fig. 4). This difference in the axis of rotation is evident in the distances between 398 and 666 and 398 and 689 in the GluR5 crystal structures; in the ATPO-bound state, these differences in distances between the antagonist-bound and glutamate-bound states are not significantly different between the two sets of residues, 5.3 and 6.7 Å, whereas in the UBP-310- and UBP-302-bound states the differences in distances are significantly different, 3.8 and 6.7 Å and 3.9 and 7.1 Å, respectively. The 3.1- and 5.7-Å distance changes between the apo and glutamate-bound states for the two sets of residues from the GluR6 LRET measurements are similar to the 3.8- and 6.7-Å distance changes observed in the GluR5 crystal structures for the UBP-310-bound state and are also not significantly different from the 3.9- and 7.1-Å distance changes observed in the UBP-302-bound state. The 3.1-Å distance change from residue 398–666 determined from the GluR6 LRET measurements of the apo state to the glutamate-bound state, however, is significantly different from the 5.3-Å distance change in the ATPO-bound state to the glutamate-bound state observed in the GluR5 crystal structures. These results suggest that the axis for rotation in the apo state of the GluR6 ligand binding domain should be similar to that observed in the UBP-310- and UBP-302-bound forms and not similar to that observed for the ATPO-bound state of GluR5 ligand binding domain, and therefore the apo state of the GluR6 ligand binding domain should be similar in structure to the UBP-310- and UBP-302-bound structures of the GluR5 ligand binding domain. The similarity of the distances in the apo state with UBP-310- and UBP-302-bound structures also implies that the cleft is as open as in the UBP-310- and UBP-302-bound structures and that the change in cleft closure upon binding agonists in GluR6 would be ~29–30°, which is one of the largest observed in this class of proteins.

Although the LRET investigations presented here probe a large range of activations, they are still not as comprehensive as that for the AMPA subtype. A detailed study such as the one performed for the GluR2 subunit (16) might reveal mutants and ligands that may not follow the general correlations between the extent of cleft closure and the extent of activation. Hence, although the cleft closure correlation holds for a number of cases, it should be noted that this may not be the only means by which the agonist controls receptor activation. A more detailed study of the dynamics of the GluR6 subunit by other spectroscopic investigations such as NMR and vibrational spectroscopy would provide more detailed insight into the various different mechanisms by which the agonists mediate receptor activation and provide insight into further similarities or differences between AMPA and kainate receptors.

**Conclusions**—The LRET investigations of the kainate subtype show that, as with AMPA receptors, the extent of cleft closure is graded and correlates to the extent of activation and suggest that the mechanism of activation of kainate receptors is similar to AMPA receptors. However, the LRET investigations in conjunction with the crystal structure studies (18, 33) suggest that the change in degree of cleft closure in kainate receptors from the apo state to the full agonist glutamate-bound states is much greater than in AMPA receptors, suggesting that although the overall mechanism of activation (cleft closure of the ligand binding domain) may be the same, the subtleties in mechanism between the two subtypes of receptors may be different.

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