Vibrational spectroscopic investigation of the ligand binding domain of kainate receptors

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Abstract: Fourier transform infrared spectroscopy has been used to probe the agonist-protein interactions in the ligand binding domain of the GluR6 subunit, one subunit of the kainate subtype of glutamate receptors. In order to study the changes in the interactions over a range of activations the investigations were performed using the wild type, N690S, and T661E mutations. These studies show that the strength of the interactions at the α -amine group of the agonist, as probed by studying the environment of the nondisulphide bonded Cys 432, acts as a switch with weaker interactions at lower activations and stronger interactions at higher activations. The α -carboxylate interactions of the agonist, however, are not significantly different over the wide range of activations, as measured by the maximum currents mediated by the receptors at saturating concentrations of agonists. Previous investigations of AMPA receptors show a similar dependence of the α -amine interactions on activation indicating that the roles of the α -amine interactions in mediating receptor activation are similar for both subtypes of receptors; however, in the case of the AMPA receptors a tug of war type of change was observed between the α -amine and α -carboxylate interactions and this is not observed in kainate receptors. This decoupling of the two interactions could arise due to the larger cleft observed in kainate receptors, which allows for a more flexible interaction for the α -amine and α -carboxylate groups of the agonists.

Keywords: glutamate receptors; glutamate; ligand protein interactions; vibrational spectroscopy; kainate receptors

Additional Supporting Information may be found in the online version of this article.

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; FTIR, Fourier transform infrared; GluR2, glutamate receptor subunit 2; GluR5, glutamate receptor subunit 5; GluR6, glutamate receptor subunit 6; GluR6-LBD, isolated ligand binding domain of GluR6; N690S-LBD, isolated ligand binding domain of GluR6 with N690S mutation; T661E-LBD, isolated ligand binding domain of GluR6 with T661E mutation; HEK 293, human embryonic kidney 293 cell line; NMDA, *N*-methyl-D-aspartate; NMR, nuclear magnetic resonance; CIW, (S)-5-chlorowillardiine; FW, (S)-5-fluorowillardiine; HW, (S)-willardiine (unsubstituted willardiine); IW, (S)-5-iodowillardiine.

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Introduction

Excitatory synaptic signals in the central nervous system are mediated primarily by ionotropic glutamate receptors.^{1–3} Based on the agonist activation profiles, ionotropic glutamate receptors have been grouped into three subtypes: kainate, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and *N*-methyl-D-aspartate (NMDA). In all three of these subtypes, glutamate binds to an extracellular ligand binding domain, initiating a series of conformational changes that ultimately result in the formation of cation specific channels in the receptor (activation). At later times the channel closes, and the receptor enters a desensitized state.^{4–6} While large conformational changes and changes in the dynamics in the receptor mediate the formation of the channel, it is through the specific noncovalent interactions between the agonist

and the extracellular ligand binding domain that these large conformational changes are initiated.^{5–9} Using vibrational spectroscopy, which possesses the sensitivity to probe the changes in the agonist-protein interactions, we have previously shown that in the GluR2 subunit of the AMPA subtype of this family of receptors, the strength of the interaction at the α -amine group of the agonist increases with an increase in the efficacy of the agonist.^{8,10,11} Additionally, the strength of the interaction at the α -carboxylate group decreases when the efficacy of the agonist increases. These changes in the strengths of the interactions for the various agonists, particularly at the α -carboxylate, have been approximately calibrated.^{8,10,12} For the AMPA receptor, the difference in the strengths of the interactions at the α -carboxylate between the full agonist glutamate and the partial agonist kainate is approximately 4.7 kcal/mol.⁸ This difference in the enthalpic contribution is significantly large and hence expected to make a sizable contribution towards the energetics. It should be noted that while these changes are large in terms of energetics, they are small in terms of length scale and cannot be observed at the resolution of X-ray structures. This decrease in the strength of the interaction at the α -carboxylate group and increase in the strength of the interaction at the α -amine group as an agonist becomes more efficacious results in a tug of war effect when various agonists bind to GluR2 and heavily influences the extent of activation by a particular agonist.^{8,10,11} While this relationship is quite evident for the AMPA subtype, the question of how the specific interactions between the agonist and the receptor influence activation, in other subtypes of glutamate receptors remains unanswered.

Here we have used vibrational spectroscopy to study the closely related kainate subtype of glutamate receptors. X-ray structures of the isolated ligand binding domains of the GluR5 and GluR6 subunits of the kainate receptor show a cleft closure conformational change upon binding agonists, with partial agonists inducing a smaller cleft closure compared to full agonists,^{13–17} similar to the results observed in the crystal structures of the isolated ligand binding domain of the GluR2 subunit of AMPA receptors.^{18–20} However, these structures also showed that the kainate receptor ligand binding domain has a significantly larger cleft than that of AMPA receptors. Additionally, it is also well established that the two receptors exhibit clear differences in their agonist binding and activation profiles.^{21–24} At present it is hence unknown if the changes in the pocket necessary for the changes in the agonist binding and activation profiles result in a change in the role of specific agonist-protein interactions in mediating receptor activation in the two receptor subtypes. To address this question, here we have used vibrational spectroscopic investigations to study the changes at the α -amine and α -carboxylate groups in the isolated ligand binding domain of the

Figure 1. Electrophysiological measurements of GluR6-flip receptors. (A) Whole cell currents mediated by 10 mM glutamate and 1 mM kainate in HEK293 cells expressing the wild type, T661E, and N690S GluR6 receptors. (B) Maximum peak currents mediated by 10 mM glutamate and 1 mM kainate for the wild type, T661E, and N690S receptors normalized to maximum currents mediated by saturating concentrations of glutamate.

GluR6 subunit of kainate receptors for the wild type, N690S and T661E mutant protein which exhibit a range of activations by the agonist kainate.^{23,24} These mutations were expected to decrease kainate efficacy based on the fact that asparagine at site 690 is a threonine and threonine at position 661 is a glutamate in the AMPA receptors subunits. The range of activation for a given agonist allowed us to determine changes in the interactions at these groups in the GluR6 subunit that could be correlated to extent of activation. These results were then compared to the previously reported changes at the α -amine and α -carboxylate groups of agonists bound to the GluR2 subunit of the AMPA receptor to see if the relationship between these interactions and activation are conserved between these two subtypes of glutamate receptors.

Results and Discussion

Functional characterization

Representative whole cell currents mediated by the wild type GluR6 flip and T661E and N690S mutants due to saturating concentrations of agonists glutamate (10 mM) and kainate (1 mM) are shown in Figure 1(A). The extent of activation for kainate relative to glutamate was determined by investigating the peak currents evoked by saturating concentrations of kainate

Figure 2. Changes at the α -carboxylate and secondary structural modes. Difference FTIR spectrum in the region of 1500 cm^{-1} to 1750 cm^{-1} between (A) glutamate and D_2O buffer (Glutamate), and (B) glutamate-bound and unligated form of GluR6-LBD ([GluR6-LBD:Glu]-[GluR6-LBD]), (C) kainate and D_2O buffer (Kainate), and (B) kainate-bound and unligated form of GluR6-LBD ([GluR6-LBD:kai]-[GluR6-LBD]).

normalized to peak currents mediated by saturating concentration of glutamate [Fig. 1(B)]. These results are in good agreement with the previously published results.^{23,24} Consistent with the previous reports the activation mediated by saturating concentrations of kainate in the wild type is approximately 50% of that mediated by glutamate. This 50% activation of GluR6 wild type receptors by kainate relative to activation by glutamate is significantly larger than the approximately 10% activation observed in the AMPA subtype.^{21,22} The ratio of kainate to glutamate mediated activation is significantly higher in the N690S mutation relative to the wild type receptor and is significantly reduced in the T661E mutation. This spread of activation by kainate on the wild type and T661E and N690S mutations provides us with an excellent system to investigate the role of specific agonist-protein interactions in mediating receptor function in kainate receptors.

Interactions between the α -carboxylate of the agonist and the protein

The difference spectra between the ligated GluR6-LBD and unligated forms for the ligands glutamate and kainate in the region of 1500 cm^{-1} to 1750 cm^{-1} are shown in Figure 2. This region of the FTIR spectrum contains

modes due to vibrations from the carboxylate moieties of the agonists. The bands arising from the asymmetric stretching mode of the α -carboxylate of the agonists have been assigned based on the frequency as well as our previous FTIR investigations using isotopically labeled ligands.^{8,25} This mode is an excellent probe for the strength of the interaction in terms of the enthalpic contributions at this moiety with a downshift in the frequency of vibration corresponding to a more favorable enthalpic interaction at this group and an upshift in the frequency of vibration corresponding to a less favorable enthalpic interaction at this group.^{8,10,11} The frequency of the asymmetric α -carboxylate vibration for glutamate in the difference spectra for GluR6-LBD is not significantly different from the frequency of this mode for glutamate in buffer (see Fig. 2), suggesting that the strength of the noncovalent interactions at this moiety in the protein is similar to that in buffer. The extent of downshift for kainate, however, is significantly large (12 cm^{-1}), indicating significantly more favorable interactions at the α -carboxylate moiety when kainate is bound in the binding pocket as compared to its interactions in buffer. Based on model compound studies this 12 cm^{-1} downshift in frequency for the bound kainate relative to kainate in buffer can be approximately correlated to a -4 kcal/mol change in the enthalpy of association at the α -carboxylate.⁸ These significantly more favorable enthalpic contributions at the α -carboxylate of kainate relative to glutamate may arise due to the fact that kainate is a bulkier ligand and is able to establish better interactions in the significantly larger cleft in the GluR6-LBD relative to glutamate.¹³

The difference spectrum between the ligated and unligated states for the wild type is compared to that for the T661E-LBD and N690S-LBD in Figure 3. These difference spectra show that there are no significant changes in the frequency of the asymmetric carboxylate stretching mode for a given ligand between the wild type and various mutant proteins. Specifically, for kainate, which exhibits a range of activations in the wild type and mutant proteins, no significant changes are observed in this mode between the kainate-bound states of the three different proteins, thus suggesting that the environment of the α -carboxylate does not exhibit any significant changes with respect to changes in activation by the agonist. It should be noted that the asymmetric stretching vibration correlates to enthalpic changes and does not provide insight into entropic changes and thus the changes in terms of free energy at the specific moieties cannot be obtained.

Secondary structural changes in the protein as a result of agonist binding

The region of the spectra shown in Figure 3 also displays amide I vibrational modes, which can be used to study the protein secondary structural changes as a result of the binding of the agonists.^{8,10,11} The amide I

Figure 3. Changes in the vibrational spectra between wild type and mutant proteins. Difference FTIR spectra in the region between agonist-bound and apo forms of GluR6-LBD, N690S-LBD, and T661E-LBD for the two agonists glutamate and kainate.

difference features observed in the spectra for the kainate-bound GluR6-LBD wild type protein are similar to the features observed for the kainate-bound T661E-LBD and N690S-LBD mutant proteins, indicating similar secondary structural changes for kainate-binding to these three proteins. This suggests that there are no changes in secondary structure that can be correlated to the changes in activation by kainate. Similarly, the amide I difference features for the glutamate-bound GluR6-LBD protein are similar to the features observed for the two mutant proteins, indicating that glutamate-binding induces similar secondary structural changes in all three proteins. When comparing the secondary structural changes for glutamate vs. kainate binding in the wild type and mutant proteins, the overall amide I difference features are different between the two agonists. Since the difference features observed in the amide I region are specific to the agonist they most likely reflect changes in the residues in close association with the ligands. Additionally, since there are no negative bands in this region these changes in the amide I region most likely do not reflect a change from one secondary structural form to another, but most likely are changes in dynamics of the secondary structural elements which would result in a change in the dipole moment and hence increase in the extinction coefficient for a given amide I transition. NMR investigations of the dynamics of the backbone for the kainate receptor would be able to shed some light on this.

Interactions at the α -amine group

Previous studies on the isolated ligand binding domain of the GluR2 subunit of AMPA receptors have established that the changes in the S—H stretching fre-

quency of Cys 425 provide insight into the strength of the interaction at the α -amine group of the agonist.^{8,10,11} Similarly, in this study with the GluR6 ligand binding domain, the S—H stretching vibration of Cys 432, which is in an equivalent position to Cys 425 in the AMPA receptor and also in close proximity to the α -amine group (see Fig. 4), was monitored to probe the strength of the interactions between the α -amine group and the protein in the glutamate- and kainate-bound states for the wild type and mutant proteins. Cys 432 is the only nondisulfide-bonded cysteine in the GluR6-LBD protein; hence the vibrational mode arising in the S—H stretching region of the spectrum can be assigned to this residue.

Figure 5 shows the FTIR spectra of the GluR6-LBD, T661E-LBD, and N690S-LBD in the apo state and glutamate- and kainate-bound states in the region of 2500–2600 cm^{-1} , which displays modes due to the S—H stretching vibration of Cys 432. The frequency of this vibration in the apo state is at 2562 cm^{-1} for GluR6-LBD and N690S-LBD and at 2565 cm^{-1} for T661E-LBD. The S—H stretching frequency in the apo state is similar to the frequencies of 2560 cm^{-1} observed for the Cys 425 residue in the apo state of the GluR2 and GluR4 subunits of AMPA receptors, suggesting that the environment of this conserved Cys residue is similar in both kainate and AMPA receptors.^{8,10,11,25}

In the glutamate-bound state, the frequency of the Cys 432 S—H stretching vibration downshifts to 2542 cm^{-1} for GluR6-LBD, T661E-LBD, and N690S-LBD. The downshifts of 20 to 23 cm^{-1} upon glutamate-binding to the wild type GluR6-LBD and N690S and T661E mutants indicate a stronger hydrogen bond at Cys 432 upon binding glutamate as compared to the apo state and suggest strong interactions at the α -amine group of glutamate in the GluR6 binding pocket in both the wild type and mutant proteins. It is

Figure 4. Binding site of GluR6-LBD (1S50).¹³ Crystal structure of glutamate-bound GluR6-LBD showing the close proximity of the single nondisulfide bonded cysteine, Cys 432, to the α -amine group of the agonist glutamate.

Figure 5. Interaction at the α -amine group as determined by Cys 432 S—H stretching mode. FTIR spectra in the region of 2500 cm^{-1} to 2600 cm^{-1} for the apo, kainate-, and glutamate-bound forms of GluR6-LBD, N690S-LBD, and T661E-LBD.

interesting to note that a similar shift of 18 cm^{-1} was also observed upon glutamate binding in the GluR2 subunit of the AMPA subtype.^{8,10} This similarity in the extent of the shift of the S—H stretching frequency indicates that in addition to the similar environments of the LBDs of the AMPA and kainate subunits in the apo state, the changes in those environments upon full agonist binding are also conserved between these two subtypes.

The frequencies of the S—H stretching vibrations for the kainate-bound states, on the other hand, exhibit differences between the wild type and mutant proteins. This band is downshifted to 2542 cm^{-1} and 2544 cm^{-1} for the GluR6-LBD and N690S-LBD relative to the S—H stretching frequencies of the corresponding apo states. However, no significant changes were observed in the frequency of the S—H stretching vibration for the T661E-LBD protein upon binding kainate relative to the corresponding apo state. The changes in the frequencies of the S—H stretching vibration when plotted as a function of activation of the agonist show a step function (see Fig. 6). A similar step function was also observed for this mode in the AMPA subtype (Fig. 6,^{8,10,11}). These results show that the α -amine interactions exhibit a correlation to the extent of activation, and that the strength of this interaction is one of the mechanisms by which the agonist can mediate receptor activation, specifically in the mid range of activations. Additionally, the similarity in

terms of the specific environment of the apo and full agonist-bound states as well the correlation between the strength of the interaction at the α -amine group and activation exhibiting a step function for both the AMPA and kainate subtypes suggests that the overall mechanism and role of specific interactions of the agonist in mediating receptor activation is conserved between these two glutamate receptor subtypes.

It is most likely that the changes studied here correspond to activation and not desensitization since the investigations are on the soluble ligand binding domain and the individual subunits are thought to primarily reflect changes in activation. Desensitization is thought to be triggered in the tetrameric structure due to the stress on the trans-membrane segments caused by the cleft closure conformational change which results in the decoupling of the dimer interface and closure of the channel.^{26–28}

Comparison between the kainate and AMPA receptors

An important aspect uncovered from the AMPA receptor studies is the tug of war relationship exhibited between the α -amine and α -carboxylate groups, where a stronger interaction at the α -amine group correlates to a weaker interaction at the α -carboxylate group for more efficacious agonists, such as glutamate and AMPA, while the reverse relationship is observed for less efficacious agonists, such as kainate.^{8,10,11} Based on the spectroscopic investigations described here for the GluR6 wild type and mutant proteins and the agonist kainate, a relationship between the extent of activation and strength of interaction at the α -amine group has been observed, where a stronger interaction at this group correlates to a larger activation of the receptor. However, these changes appear to be

Figure 6. Correlation between α -amine interactions and extent of activation. Normalized activation of wild type GluR6-flip receptors, T661E and N690S (as measured by whole cell current recordings and shown in Figure 1) plotted as a function of the frequency of the Cys 432 S—H stretching mode along with previous data obtained for the GluR2 subunit of AMPA receptors.^{8,10,11}

unaccompanied by changes in the strength of the interaction α -carboxylate group, as the vibrational frequency for this moiety is the same for kainate bound to the wild type and N690S and T661E mutant proteins, despite the variation in activation by kainate in the full receptor for the wild type and two mutants. Thus, it appears that the tug of war relationship observed between the α -carboxylate and α -amine groups of the agonists for AMPA receptors^{8,10,11} is not the case for the kainate subtype.

The crystal structures of the GluR6-LBD show that the cleft is 16% larger than that of GluR2.¹³ Hence it is possible that the larger size of the cleft results in greater conformational flexibility in the GluR6 binding pocket and consequently an absence of a tug of war relationship between the α -carboxylate and α -amine groups. Future investigations using NMR and possibly single molecule methods should provide insight into further differences between the AMPA and kainate receptors which would shed light on the structural differences in these two receptors in terms of dynamics of the two proteins. Differences in dynamics between the AMPA and kainate subtypes could contribute to the differences in the rates of activation and desensitization observed between these two subtypes of the glutamate receptor.^{2,6,13–17} While, the two receptors still maintain primarily a similar pathway for the allosteric mechanism by which agonists mediate receptor activation.

Materials and Methods

Chemicals

Glutamate was purchased from Sigma-Aldrich (St. Louis, MO). Kainate was purchased from Ascent Scientific (Somerset, England, UK), and [³H]-kainate was purchased from Perkin Elmer (Waltham, MA).

Preparation and purification of GluR6-LBD

The GluR6-flip plasmid was kindly provided by Dr. Kathryn Partin (Colorado State University, Fort Collins, CO), and the GluR6-LBD plasmid was constructed for expression of the wild type GluR6-LBD protein as previously described.¹³ The T661E and N690S mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Generation of the constructs and mutations were confirmed by DNA sequencing. The GluR6-LBD, T661E-LBD, and N690S-LBD proteins were expressed, purified, and characterized as previously described.¹³ Briefly, the proteins were expressed in *Escherichia coli* Origami-B (DE3) cells, and purified by a Ni-NTA HiTrap affinity column (GE Healthcare Life Sciences, Piscataway, NJ) followed by thrombin overnight digestion to remove the histidine tag. Anion exchange chromatography using Q-Sepharose Fast Flow resin (GE Healthcare Life Sciences) was then used for further

purification. The functionality of all purified proteins was established by the saturation binding analysis of [³H]-kainate to the proteins (Supporting Information Figure).

FTIR difference spectroscopy

The protocol outlined in Refs. 7,8,11 was used to obtain the FTIR difference spectra of bound and unbound GluR6-LBD, T661E-LBD, and N690S-LBD. For the FTIR investigations, 0.8–1.2 mM protein in 25 mM phosphate buffer with 250 mM NaCl and 0.02% NaN₃ (pH 7.4) or 10 mM HEPES buffer containing 100 mM NaCl (pH 7.0) was used. For spectra in the region of 1450–1800 cm⁻¹, D₂O was used as the solvent to obtain spectra due to the large infrared absorption of water at $\approx$ 1600 cm⁻¹. To study the S–H stretching vibration, water was used as the solvent.

A Nicolet Nexus 870 spectrometer was used to collect FTIR spectra. Spectra were obtained using an adjustable path length infrared cell holder (Aldrich). To obtain spectra in the 1450–1800 cm⁻¹ region, a path length of 50 μ m was used while for the S–H stretching investigations, a path length of 75 μ m was used. Spectra were collected at 4 cm⁻¹ resolution and a constant temperature of 15°C. Difference spectra were obtained by subtracting the spectrum collected for the unligated form of the protein from the spectrum obtained for the ligated form of the protein. Peaks arising from unbound agonists were subtracted using a spectrum of the agonist in buffer. Data analysis and collection were performed using Omnic and GRAMS/AI (7.01) software (ThermoFisher, Waltham, MA).

Electrophysiological measurements

The wild type and mutant receptors were expressed in human embryonic kidney 293 (HEK 293) cells (ATCC CRL 1573), and the cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Transfections were performed using Fugene-6 (Roche, Indianapolis, IN) transfection reagent using 1–2 μ g of GluR6 cDNA and 0.2–0.4 μ g of cDNA for green fluorescent protein. Experiments with the transfected cells were performed 2–3 days after transfection.

To obtain whole cell electrophysiological current recordings, HEK 293 cells transfected with appropriate GluR6 receptors were voltage-clamped at a holding potential of –60 mV, and solutions were applied to the cells using a homemade U-tube mixing device with a 100 μ m aperture. The electrode solution for the recordings consisted of 140 mM CsCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, 2 mM Na₂ATP and 10 mM HEPES (pH 7.4), and the extracellular bath solution consisted of 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 (Molecular Devices, Sunnyvale, CA) and low

pass filtered at 1 kHz. The filtered signal was then digitized using a Labmaster DMA digitizing board that was controlled by Axon PClamp software (Molecular Device, Sunnyvale, CA). Experiments were performed at room temperature.

Conclusions

Previous vibrational spectroscopic studies of the isolated ligand binding domain of the GluR2 subunit of AMPA receptors have established the importance of specific interactions in the binding pocket that are important in mediating activation in these receptors.^{8–11} Here we have used vibrational spectroscopy to analyze the specific agonist-protein interactions important for activation in kainate receptors. As with the AMPA receptors, the strength of the interaction at the α -amine group correlated with the extent of activation of the receptor, with a stronger interaction leading to a larger activation. However, the tug of war relationship between the α -carboxylate and α -amine groups of the agonist exhibited in AMPA receptors was not observed in kainate receptors, possibly due to the larger size of the binding cleft in kainate receptors. These investigations show that while certain interactions are conserved between the two subtypes, others are not, suggesting that other subtle mechanisms distinguish the two receptor subtypes from each other.

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