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THE ROLE OF THE C-4 SIDE CHAIN OF KAINATE AND DIHYDROKAINATE IN EAA RECEPTOR AND TRANSPORTER SELECTIVITY

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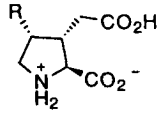
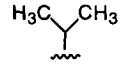
Abstract: Molecular modeling was used evaluate conformational effects of side chain modifications to kainate and the pharmacological consequences of such modifications on binding to KA, NMDA, and AMPA receptors and to the high-affinity sodium-dependent glutamate transporter. Copyright © 1996 Elsevier Science Ltd

L-Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS), participating in both standard fast synaptic signaling and the more complex forms of neuronal communication required in higher cognitive function.[1] Glutamate and related excitatory amino acids (EAAs) activate at least five distinct classes of receptors, which have been distinguished based on the pharmacological action of selective agonists and antagonists.[2] Both synthetic and naturally-occurring conformationally restricted glutamate analogs have played a key role in categorizing the receptor classes and defining a pharmacophore specific to each. Among these analogs, kainic acid has been instrumental in demonstrating the existence, physiological properties, and anatomical distribution of one such glutamate receptor, which is indeed named after the natural product (KA).

Given the structural similarities between kainate and glutamate, considerable interest has focused on the influence of the isopropenyl side group in determining kainate's specificity of action. Previous studies have demonstrated that when this C-4 appendage is fully saturated (i.e., dihydrokainate; DHK), activity as a KA receptor agonist is diminished while activity as an inhibitor of the sodium-dependent glutamate transporter increases. Interestingly, the corresponding analog lacking a substituent, 2-carboxy-3-pyrrolidineacetic acid (CPAA), exhibits very different binding selectivity, namely activity as an NMDA receptor agonist.[3] In the present study we have directly compared the ability of KA, DHK, and CPAA to bind to KA, AMPA, and NMDA receptors as well as to the sodium-dependent high affinity glutamate transporter. Based on these data and molecular modeling studies, we offer a rationale for the conformational and steric control of binding selectivities.



Kainic Acid R= 

Dihydrokainate R= 

CPAA (2-carboxy-3-pyrrolidineacetic acid) R= 

Receptor and Transporter Pharmacology

Synaptic plasma membranes (SPM's) were prepared from rat forebrain and assayed for radioligand receptor binding as described previously.[4] Estimates of K_is were made with these IC_{50} values using the method of Cheng and Prusoff;[5] $K_i = IC_{50}/(1+L/K_d)$ with K_d values of 300 nM, 45 nM, and 9 nM for glutamate, kainate and AMPA, respectively. Rat forebrain synaptosomes were prepared from Sprague-Dawley rats as described previously.[6] Lineweaver-Burk plots and associated kinetic analysis of the transport inhibitors were carried out using k•cat kinetic program (BioMetallics Inc.) with weighting based on constant relative error. K_i values were estimated on the basis of a replot of $K_{m,app}$ values from 3-5 experiments.

Results and Discussion:

Binding studies (Table) with DHK confirm that saturation of the isopropenyl side chain of KA results in an inhibitor of high-affinity ^{3}[H]-KA binding that is weaker by a factor of more than 1,000. DHK also exhibited little or no cross-reactivity with AMPA and NMDA receptors, but consistent with previous studies proved to be a moderate inhibitor of the sodium-dependent glutamate transporter (K_i = 30 μM). At the other extreme, the analog lacking a side chain at C-4 (CPAA) only partially retains its ability to bind to the KA receptor; the K_i for CPAA is more than 100-fold greater than for kainate itself. CPAA was also distinct from kainate in the extent of its cross reactivity with the other EAA receptors. Thus, while both kainate and CPAA inhibited ^{3}[H]-KA and ^{3}[H]-AMPA binding, kainate exhibited a much greater degree of discrimination between the two sites (i.e., ≈1,200-fold for kainate vs. ≈ 60-fold for CPAA). Transport assays demonstrated that the lack of this side chain also results in a marked reduction of the inhibitory activity exhibited by both DHK and kainate at the sodium-dependent, synaptosomal glutamate transporter. Most significant, however, was the increased affinity of CPAA for the NMDA receptor: when compared to kainate, CPAA was more than 600-fold more potent as an inhibitor of ^{3}[H]-glutamate binding at the NMDA site. Put another way, complete "removal" of the kainate isopropenyl side chain alters the activity from high potency at the KA receptor to greatest potency at NMDA receptor, while increasing its steric bulk tips the balance in favor of greatest affinity for the transporter.

Table. *K_i values for inhibition of radioligand binding and transport**

Compound	^{3}H-Kainate Binding to KA Receptors (μM)	^{3}H-AMPA Binding to AMPA Receptors (μM)	^{3}H-L-Glutamate Binding to NMDA Receptors (μM)	^{3}H-D-Aspartate Uptake into Synaptosomes (μM)
KA	0.01	12	>300	59
DHK	16	>500**	>300**	30
CPAA	1.6	106	0.5	>250**

**K_i values in the binding assays were estimated from the IC_{50} values determined in dose response curves, while K_i values for the transport assays were estimated from a replot of $K_{m,app}$ values determined by Lineweaver-Burk plots. In instances of very weak inhibition (**), K_i values were estimated from the level of inhibition produced from a single high concentration of analog.*

There are numerous discussions in the literature about the cause of differences in binding specificities between kainate and DHK. Generally, the focus has been on the size of the C-4 side chain, and the following statement typifies the type of steric argument often put forth: "the C1'-C2' double bond confers a planar 'knife-blade' shape to this part of the side chain that appears to be necessary for high affinity binding...When the first double bond is reduced...the planar shape is replaced by a nonplanar 'umbrella' shape that occupies much more volume, thus interfering with binding...".[7] While it certainly has been recognized that conformational effects may also come into play,[7,8] we sought to identify the specific conformations responsible for the fascinating divergence of binding selectivities among this group of compounds. Although direct steric inhibition of binding appears to be important in a few instances, biasing of conformational preferences by the C-4 side chain appears to play an even more important role in controlling binding selectivities.

Molecular Modeling

Molecular modeling was performed on a Silicon Graphics Indigo 2 workstation using the BIOSYM molecular modeling software package. Conformational searches were carried out using a modified AMBER forcefield[9] and were conducted as follows: first, a local minimum was found by application of 1000 steps of a

conjugate gradient minimization until the RMS deviation was less than 0.001 Å. Conformational space was then explored by use of a dynamics simulation where the molecule is effectively subjected to 600 K for 500.000 iterations at 1 fs intervals. The molecular geometry of the compound was recorded after every 5000 fs, yielding a total of 100 high energy geometries. These high energy geometries were then minimized using conjugate gradients minimization as above. Unique conformations with energies within 3 kcal of the lowest energy conformer were deemed significant and saved. The molecules were modeled as having a positively charged N atom and two negatively charged carboxylates, as would exist at approximately neutral pH; the dielectric constant was set at $\varepsilon = 80$. Comparisons of different compounds were done using an overlay method in which three points of comparison (the amine functionality, the proximal carboxylate, and the distal carboxylate/anionic center) were specified for superimposition. The lowest RMS deviation was taken as the best fit, with all successful overlays falling within 0.35 Å. In order to define receptor pharmacophores, known agonists for each receptor were subjected to conformational searches to determine the low energy conformations available to each agonist. The rigid agonists were then overlayed, and positional averages were calculated for each of the three comparison points; these averages were then used as comparison points for all subsequent overlays. This resulted in a structure that had a common arrangement of functional groups and that defines the pharmacophore of a particular receptor. The preferred arrangement of functional groups for each pharmacophore is represented below (Figure 1) by the corresponding all-staggered conformation of L-glutamate.[10]

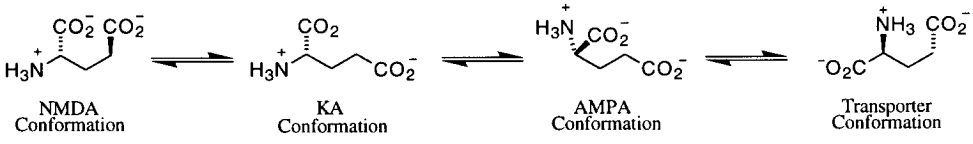


Figure 1. Calculated glutamate conformations representing EAA receptor pharmacophores

Initial modeling studies revealed distinct preferences in ring conformation among KA, DHK, and CPAA (Figure 2). The lowest energy DHK conformer adopts a 3-exo ring conformation, while the 3-endo conformation is preferred by the other two molecules (exo refers to the ring flap being on the opposite face of the ring as the α-carboxylate);[11] this effect can be attributed to the large steric bulk of the isopropyl appendage, which must be deployed pseudo-axially in the 3-exo conformation. In addition, though the smaller isopropenyl group does not have as pronounced an effect on ring conformational preferences as does the isopropyl group, its steric bulk does influence the rotamer populations of the adjacent acetate appendage; the lowest energy KA and CPAA conformers differ considerably in respect to rotation of the C-3 acetate appendage.

These studies clearly suggest that the identity of the C-4 side chain does have a significant effect on biasing the conformations of these three compounds.[12] To determine how these conformational preferences might relate more specifically to binding affinity, all calculated low-energy conformations of DHK, KA, and CPAA (Figure 2) were overlayed with each pharmacophore (Figure 3, calculated as described above) to determine which conformations fit each. Measured binding affinities were then compared with the calculated receptor preferences based on the assumption that a better fit with the pharmacophore by a low energy conformation should correlate with higher affinity for that receptor. In some cases, the fit was good for an observed poor binder; however, in all of these instances, the C-4 appendage diverged considerably from other conformers that overlayed well at that pharmacophore, suggesting an excluded volume that cannot be accommodated in the binding site. These outlying conformations are represented in gray to emphasize conformers that do not bind -- despite a good fit -- because of

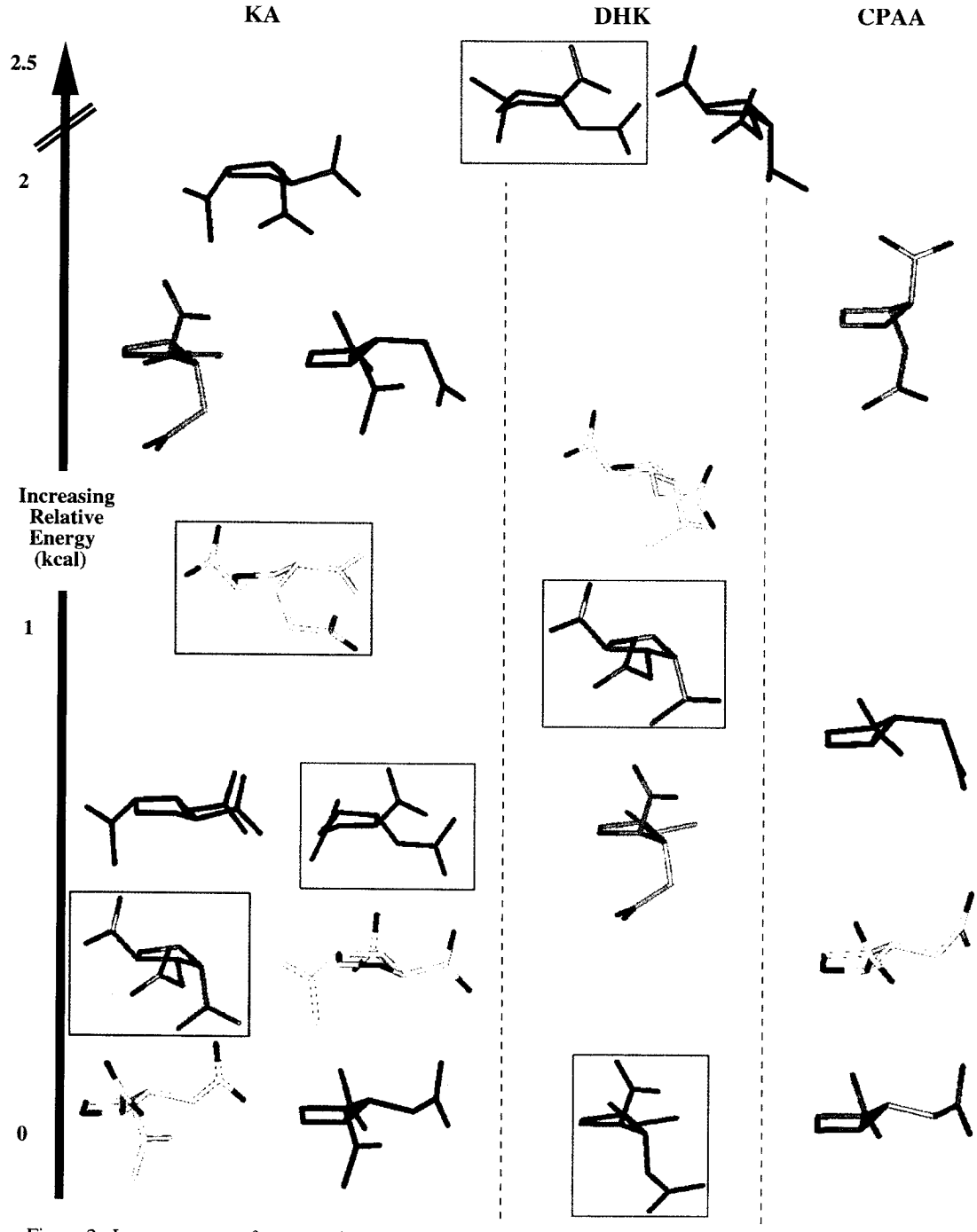


Figure 2. Low energy conformers calculated for each molecule, color-coded to indicated the pharmacophore at which good overlays were obtained: =KA; Pink=Transporter; Orange=NMDA; Purple=AMPA; Green= NMDA and AMPA; Black=no good fit found. Outliers (see text) are outlined in a gray box.

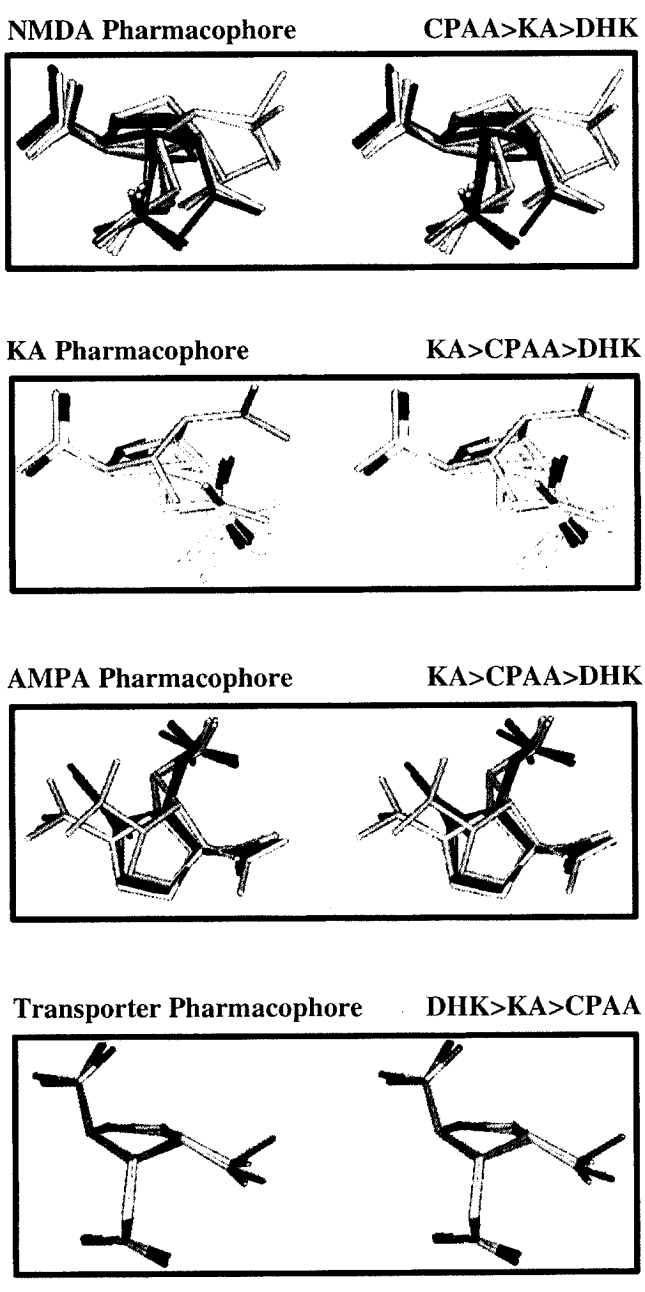


Figure 3. Overlays at receptor pharmacophores, color-coded to indicate the pharmacophore preferred: =KA; Pink= Transporter; Orange=NMDA; **Purple**=AMPA; **Green**=NMDA and AMPA; **Gray**=Outlier (see text).

direct steric effects. For example, the conformers of KA, DHK, and CPAA were first overlayed with the NMDA pharmacophore. Eight conformations were calculated to give a good fit with this pharmacophore: four KA, two DHK, and two CPAA conformers (Figure 3). Of these, both DHK conformers and the two lower energy KA conformers were excluded based on the positioning of the C-4 appendage. The lowest energy CPAA conformer with a good fit at NMDA is actually the lowest energy CPAA conformer, while the KA conformers are the two highest energy KA conformers found. Based on overlays and the relative energies of the conformers, CPAA would be predicted to be most active at NMDA, while KA should be somewhat active and DHK completely inactive; this order correlates well with the binding data. Five conformers (three KA and one each of DHK and CPAA) were found to overlay well with the KA pharmacophore. Once the fit of one of the KA conformers was discounted due to excluded volume as described above, the resultant overlays correlated well with the measured binding data. The ground state conformer of KA is expected to give the best fit at KA, followed by the low energy (0.3 kcal) CPAA conformer and the higher energy (1.2 kcal) DHK conformer.

Though six conformers (three of KA, two of DHK, and one of CPAA) were calculated to give a good fit with the AMPA pharmacophore, one KA and both DHK conformers are outliers with excluded volumes. The measured affinity of KA and CPAA for AMPA is consistent with the remaining calculated 'active' conformers. However, the relative energies of the KA and AMPA conformers are opposite to the

observed binding affinities, a reminder that the conformational arguments presented here do not account for all of the factors involved in receptor binding. Finally, one conformer of each analog gave a reasonable fit with the transporter pharmacophore. In this case, the relative energies of the conformers again correlated with the measured binding affinities: DHK was lowest in energy (and exhibited strongest binding), followed in order by the KA conformer and the CPAA conformer.

The modeling results, taken together with the binding data, suggest which specific conformations of KA, DHK, and CPAA are preferred by each EAA receptor, as well as identifying excluded volumes that mitigate binding in some cases. It is clear that members of the kainate family can bind to a given receptor only in appropriate conformations, and these studies have shown that conformational biasing by the C-4 appendage strongly influences which conformations are accessible.

Acknowledgments

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10. Agonists used to define the NMDA receptor: trans-ACBD, (2R,3S,4R)-CCG, trans-2,3-PDC; KA receptor: (1R,3S)-ACPD, (1S,3S)-ACPD; AMPA receptor: 7-HPCA, AMPA, QA; Glutamate transporter: cis-ACBD, (2S,3S,4R)-CCG, trans-2,4-PDC. The pharmacophores shown are consistent with those proposed previously (for a summary, see reference 2). A more folded form has recently been proposed for the KA pharmacophore (Shimamoto, K.; Ohfune, Y. *J. Med Chem.* **1996**, *39*, 407), although the pharmacophores proposed there for the other receptors are consistent with those in Figure 1.

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12. The solution ^{1}H NMRs clearly indicate very different ring conformer populations in solution; the H2-H3 coupling constants for DHK, KA, and CPAA are 0, 3.2, and 7.5 Hz, respectively.

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